University of Alberta

Effect of ammonia load on glucose metabolism by isolated ovine duodenal mucosal cells

by

Prajwal Raj Regmi ©

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science

in

Animal science

Department of Agricultural, Food and Nutritional Science

Edmonton, Alberta
Fall 2007

Library and Archives Canada

Published Heritage Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et Archives Canada

Direction du Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 978-0-494-33332-7
Our file Notre référence
ISBN: 978-0-494-33332-7

NOTICE:
The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:
L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

University of Alberta

Library Release Form

Name of Author: Prajwal Raj Regmi

Title of Thesis: Effects of ammonia load on glucose metabolism by isolated ovine duodenal mucosal cells

Degree: Master of Science

Year this Degree Granted: 2007

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as herein before provided, neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatsoever without the author's prior written permission.

Signature

521 M, Michener Park,
Edmonton, AB T6H 4M5

Dated: May 3, 2007

ABSTRACT

Greater intestinal glucose supply can increase the productivity of high yielding dairy cows in early lactation. Glucose metabolism in the duodenal mucosal cells (DMC) affects its delivery to the peripheral circulation. This study evaluated the effects of ammonia load on glucose metabolism in the DMC isolated from sheep fed either a high or low crude protein (CP) diet. The isolated DMC were incubated for 90 min with 3 m*M* glucose and varying concentrations of ammonia in Krebs-HEPES buffer. Enhanced ammonia load decreased the utilization of glucose carbon for alanine synthesis but had no effect on glucose carbon utilization for aspartate or glutamate synthesis. Glucose disappearance, however, was greater for the DMC isolated from sheep fed a low vs. high CP diet. These observations indicate that enhanced ammonia load does not increase glucose utilization by DMC, and that glucose carbon may not play a significant role in assimilation of ammonia-N into amino acids.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor, Dr. Masahito Oba for providing me an opportunity for graduate study. His expertise, support and invaluable guidance made my experience as a Masters student worthwhile. I would also like to thank Dr. Walter Dixon, my advisory committee member, for his encouragement, guidance and intellectual contributions to my research study.

I am grateful to Dr. Brian Bequette at the University of Maryland for providing me an opportunity to learn GC-MS technique. I appreciate Nishanth Sunny and Sandy Owens at Dr. Bequette's lab, for their assistance in GC-MS sample analysis. I would like to thank Steve Melnyk, Charlene Gorsak, Brenda Tchir, Clint Lysgaard, Randy O'Hara and Paul Zalkovic at the Edmonton Research Station for their help in sample collection. I also appreciate the help of Jessica Bilobrowka, Layne Manson, Letitia Chow, Catarina Silveira and Greg Penner.

I acknowledge the cooperation of Edmonton Custom Packers for providing duodenal samples. I am thankful to John Turchinsky, Urmila Basu and Lynn Elmes for their assistance in handling laboratory equipments.

I would like to acknowledge Natural Science Engineering Research Council of Canada for providing financial support for this study.

Finally, I would like to thank my wife, Bina for her love and support throughout the journey.

TABLE OF CONTENTS

CHAPTER 1. General introduction 1
1.1 References 4

CHAPTER 2. Review of the literature 6
2.1 Nutrient utilization in ruminant animals 6
2.1.1 Differences between ruminant and non-ruminant animals 7
2.1.2 Energy supply in ruminants 9
2.1.3 Glucose supply in ruminants 10
2.1.4 Splanchnic metabolism 10
2.2 Starch digestion in ruminant small intestine 11
2.3 Glucose metabolism in the small intestine 13
2.3.1 Glucose availability 13
2.3.2 Glucose absorption in the intestinal mucosa 14
2.3.3 Glucose metabolism pathways in DMC 15
2.3.4 Effects of CP on glucose metabolism by intestinal mucosa 18
2.3.5 Activity of enzymes involved in glucose metabolism 19
2.4 Volatile fatty acids metabolism in the gut tissues 20
2.5 Nitrogen metabolism in the gut tissues 22
2.5.1 Glutamine 23
2.5.2 Glutamate 23
2.5.3 Other non-essential amino acids 24
2.5.4 Essential amino acids 25
2.5.5 Ammonia 26
2.6 Limitations of in vitro metabolism studies 27
2.7 Conclusion 29
2.8 References 31

CHAPTER 3. Evaluation of procedures for the isolation of ruminant enterocytes 47
3.1 Introduction 47
3.2. Materials and Methods 48
3.2.1 Collection of duodenal segments 48

3.2.2 Duodenal mucosal cell isolation 48
3.2.2.1 Procedure A 49
3.2.2.2 Procedure B 50
3.2.2.3 Procedure C 50
3.2.3 Viability determination and cell counting 51
3.2.4 Statistical analysis 52
3.3. Results and discussions 52
3.4 References 54

CHAPTER 4. Effect of ammonia load on glucose metabolism by isolated ovine duodenal mucosal cells 58
4.1 Introduction 58
4.2 Materials and methods 59
4.2.1 Animals and diets 59
4.2.2 Ammonia measurement 60
4.2.3 Cell isolation 61
4.2.4 Incubation and metabolite analysis 62
4.2.5 Enzyme assay 65
4.2.6 Statistical analysis 65
4.3 Results 66
4.4 Discussion 67
4.5 References 72

CHAPTER 5. General discussion and conclusion 83
5.1 General discussion 83
5.2 Future research 85
5.3 Conclusion 87
5.5 References 88

APPENDIX 90
A 1. Enzymatic assay of pyruvate kinase 90
A 2. Enzymatic assay of hexokinase 92
A 3. Ammonia-N determination 95
A 4. Glucose determination 97

LIST OF TABLES

Table 4.1 Ingredients and nutrient composition of the experimental diets.... 77

Table 4.2 Effects of dietary CP level on the performance of growing sheep 78

Table 4.3 Effects of dietary CP level on glucose utilization by isolated duodenal mucosal cells in vitro 79

Table 4.4 Effects of ammonia concentration in the incubation media on glucose utilization by isolated duodenal mucosal cells in vitro..... 80

LIST OF FIGURES

Figure 2.1 Major possible pathways for glucose metabolism in the gut tissues 17

Figure 3.1. Number of viable DMC obtained immediately after slaughter per 50-cm long bovine duodenal segment in different isolation procedures ...56

Figure 3.2. Viability of bovine DMC immediately and six hours after isolation by different isolation procedures 57

Figure 4.1. Hexokinase activity in the duodenal mucosa of growing female sheep fed low (13.1%) and high (19.4 %) CP diets.....81

Figure 4.2. Pyruvate kinase activity in the duodenal mucosa of growing female sheep fed low (13.1%) and high (19.4 %) CP diets.....82

LIST OF ABBREVIATIONS

ADF	Acid detergent fiber
ADG	Average daily gain
BSA	Bovine serum albumin
BW	Body weight
CoA	Coenzyme A
CP	Crude protein
d	Day
DM	Dry matter
DMC	Duodenal mucosal cells
DMI	Dry matter intake
EAA	Essential amino acids
EDTA	Ethylene diamine tetra acetate
FAPWC	Faculty Animal Policy and Welfare Committee
GI	Gastro intestinal
GLUT 2	Glucose transporter 2
HEPES	4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid
KRB	Krebs Ringer Buffer
MJ	Mega Joule
MDV	Mucosal drained viscera
MP	Metabolizable protein
NDF	Neutral detergent fiber
NEAA	Non-essential amino acids
NE_L	Net energy of lactation
NRC	National Research Council
PDV	Portal drained viscera
RDP	Rumen degradable protein
SGLT 1	Sodium glucose co-transporter 1
VFA	Volatile fatty acids

CHAPTER 1

GENERAL INTRODUCTION

Adequate glucose supply in early lactation is important to ensure better production and reproductive performances in high producing dairy cows. Glucose supply from gluconeogenesis alone may not be sufficient to meet the high glucose demand for milk lactose synthesis (Hurtaud et al., 2000). Intestinal glucose supply is useful to compensate for the glucose shortage in lactating dairy cows because it was observed that greater intestinal glucose supply increased milk synthesis in dairy cows (Rigout et al., 2002). In ruminant animals fed a high starch diet, intestinal glucose absorption is greater and the absorbed glucose can contribute up to 30% of the total glucose supply (Huntington, 1997).

The gastrointestinal (GI) tract plays a vital role in nutrient delivery to the peripheral tissues. Within the GI tract, the duodenal mucosa is an important tissue for nutrient digestion and absorption. Depending upon dietary and physiological factors, the duodenal environment is variable, which can affect glucose metabolism in the mucosa and its net absorption across the portal drained viscera (PDV). For example, greater dietary crude protein (CP) decreases glucose utilization in the gut mucosal cells and increases net glucose absorption across the PDV in ruminant animals (Richards et al., 2002; Taniguchi et al., 1995). Similarly, the presence of other metabolites such as

propionate, butyrate, glutamate and glutamine affects glucose metabolism in the duodenal mucosa in vitro (Oba et al., 2004).

Previous studies indicated that ammonia-N can be assimilated into alanine, glutamine, glutamate, aspartate and citrulline by the duodenal mucosal cells (DMC; Oba et al., 2005; Wu, 1998). As glutamate, glutamine and aspartate are extensively catabolized and not net-absorbed across the PDV (Wu, 1998), ammonia-N assimilation into alanine or citrulline are more useful pathways to enhance the systemic delivery of amino-N in the body. The absorption of amino-N from the intestine instead of ammonia-N is beneficial in two ways. Firstly, more amino-N is available to the animal and, secondly, the hepatic catabolism of amino acids for urea synthesis is reduced (Lobley et al., 1995) because less ammonia-N is present in the liver for detoxification. Increasing absorption of amino-N instead of ammonia-N is a very useful strategy to improve the nitrogen utilization efficiency in ruminant animals.

Ammonia-N assimilation into alanine by ruminant DMC increased in the presence of glucose in vitro (Oba et al., 2005). However, the role of glucose carbon in amino acid synthesis by ruminant DMC in higher ammonia concentration is still not clear. Glucose metabolism in the duodenal mucosa and net glucose absorption across the PDV at varying ammonia concentration has not been studied yet.

Isolated DMC are required for in vitro experiments on the metabolism of duodenal mucosa. Isolated small intestinal mucosal cells have been used to study metabolism in the intestinal mucosa of rats, chickens (Watford et al., 1978), guinea pigs (Gross et al., 1986) and swine (Wu, 1995). The isolation procedures of mucosal cells have already been established in these species. However, the use of isolated intestinal mucosal cells is a relatively recent practice in intestinal metabolism studies in ruminant animals. There are very few studies (Oba et al., 2004; Okine et al., 1995) using isolated DMC in ruminant animals, and the isolation procedures described by these authors have not been evaluated by others.

The objectives of this research study are to 1) evaluate the efficacy of isolation procedures previously reported (Oba et al., 2004; Okine et al., 1995; Wu, 1995) for the isolation of ruminant DMC, and 2) evaluate the effects of ammonia load in the incubation media and those of dietary CP content on glucose metabolism in the DMC isolated from growing sheep.

1.1 References

Gross, C. J., L. M. Henderson, and D. A. Savaiano. 1986. Uptake of L-carnitine, D-carnitine and acetyl-L-carnitine by isolated guinea-pig enterocytes. Biochim. Biophys. Acta. 886:425-433.

Huntington, G. B. 1997. Starch utilization by ruminants: From basics to the bunk. J. Anim. Sci. 75:852-867.

Hurtaud, C., S. Lemosquet, and H. Rulquin. 2000. Effect of graded duodenal infusions of glucose on yield and composition of milk from dairy cows. 2. Diets based on grass silage. J. Dairy Sci. 83:2952-2962.

Lobley, G. E., A. Connell, M. A. Lomax, D. S. Brown, E. Milne, A. G. Calder, and D. A. Farningham. 1995. Hepatic detoxification of ammonia in the ovine liver: Possible consequences for amino acid catabolism. Br. J. Nutr. 73:667-685.

Oba, M., R. L. Baldwin VI, and B. J. Bequette. 2004. Oxidation of glucose, glutamate, and glutamine by isolated ovine enterocytes in vitro is decreased by the presence of other metabolic fuels. J. Anim. Sci. 82:479-486.

Oba, M., R. L. Baldwin VI, S. L. Owens, and B. J. Bequette. 2005. Metabolic fates of ammonia in ruminal epithelial and duodenal mucosal cells isolated from growing sheep. J. Dairy Sci. 88:3963-3970.

Okine, E. K., D. R. Glimm, J. R. Thompson, and J. J. Kennelly. 1995. Influence of stage of lactation on glucose and glutamine metabolism in isolated enterocytes from dairy cattle. Metabolism. 44:325-331.

Richards, C. J., A. F. Branco, D. W. Bohnert, G. B. Huntington, M. Macari, and D. L. Harmon. 2002. Intestinal starch disappearance increased in steers abomasally infused with starch and protein. J. Anim. Sci. 2080:3361-3368.

Rigout, S., S. Lemosquet, J. E. Van Eys, J. W. Blum, and H. Rulquin. 2002. Duodenal glucose increases glucose fluxes and lactose synthesis in grass silage-fed dairy cows. J. Dairy Sci. 85:595-606.

Taniguchi, K., G. B. Huntington, and B. P. Glenn. 1995. Net nutrient flux by visceral tissues of beef steers given abomasal and ruminal infusions of casein and starch. J. Anim. Sci. 73:236–249.

Watford, M., P. Lund, and H. A. Krebs. 1978. Isolation and metabolic characteristics of rat and chicken enterocytes. Biochem. J. 178:589-596.

Wu, G. 1995. Urea synthesis in enterocytes of developing pigs. Biochem. J. 312:717-723.

Wu, G. 1998. Intestinal mucosal amino acid catabolism. J. Nutr. 128:1249-1252.

CHAPTER 2

REVIEW OF THE LITERATURE

2.1 Nutrient utilization in ruminant animals

Ruminant animals are capable of utilizing fibre and non-protein nitrogen, but are less efficient in digestion and absorption of protein and starch because of microbial fermentation in the rumen. Organic matter ferments to volatile fatty acids (VFA), mainly acetate, propionate and butyrate, and produces CH_4, CO_2, H_2O and energy (Allen, 1982; Fahey and Gerger, 1988). Rumen microbes use the energy produced from fermentation for their growth and maintenance. End products of organic matter fermentation in the rumen not utilized by rumen microbes are absorbed into the portal vein. Acetate is primarily oxidized throughout the body to generate energy and utilized for the production of acetyl Co-A for lipid synthesis, and a very small amount is metabolized by the liver (Bergman and Wolff, 1971). Approximately 80 - 100% of the propionate and butyrate absorbed into the portal vein is metabolized by the liver (Bergman and Wolff, 1971). Propionate serves as one of the major substrates for gluconeogenesis in the liver. Depending on the diet, propionate supplies 50 - 75% of the glucose requirements of ruminant animals (Bergman et al., 1966). Approximately 90% of the butyrate absorbed into the ruminal epithelial cells is converted to ketone bodies, mainly β-hydroxybutyrate and acetoacetate, prior to their release into the portal vein (Weigand et al., 1975). The ketone bodies are oxidized in many tissues for energy production. Escaped starch is digested

in the small intestine and absorbed as glucose. However, Huntington et al. (2006) observed that starch digestion and absorption in the ruminant small intestine is limited probably because of the insufficient secretion of amylase (Huntington, 1997) or limited activity of the sodium glucose co-transporter (SGLT) 1 (Bauer et al., 2001; Rodriguez et al., 2004).

Similarly, dietary protein is digested in the rumen to peptides, amino acids, keto-acids and ammonia, and used for microbial protein synthesis. Ruminant animals derive 40 - 70% of amino acid requirement from rumen microbial protein (Clark et al., 1992). Excess ammonia is later converted to urea in the liver (Huntington, 1989) and the gut epithelial cells (Oba et al., 2004b; Wu, 1995). Microbial and escape protein are digested and absorbed in the small intestine as amino acids. Likewise, digestion and absorption of fat primarily occurs in the small intestine as in non-ruminant animals.

2.1.1 Differences between ruminant and non-ruminant animals

One of the major differences between ruminant and non-ruminant animals is the availability of glucose and source of energy. In lactating ruminant animals, gluconeogenesis in the liver represents 75% or more of the total glucose entering blood stream (Reynolds et al., 2003). The primary substrates for gluconeogenesis in the liver are propionate (55 - 58%) followed by lactate (18 - 21%), glucogenic amino acids (15-17%) and glycerol (0.4%) (Reynolds et al., 1988). In the case of non-ruminant animals, however, absorption of

monosaccharides resulting from enzymatic digestion of dietary carbohydrates constitutes a major source of blood sugar. Other minor sources of glucose are endogenous synthesis from glucogenic amino acids, glycerol, and propionic acid (Allen, 1982).

Another important difference between ruminant and non-ruminant metabolism arises from the availability and role of acetate and glucose, as precursors of acetyl-CoA for later use in oxidation or for synthetic processes such as lipogenesis. Ruminant animals absorb more acetate but far less glucose into the blood than do non-ruminant animals. The VFA supply 72 - 78% and up to 25% of whole body energy requirement in ruminant and non-ruminant animals, respectively (Bergman, 1990). Ruminant animals utilize acetate instead of glucose as a major substrate for energy storage and oxidation in the fed state (Schingoethe et al., 1988). Only a small proportion of absorbed acetate is utilized by the liver in ruminant animals (Bergman and Wolff, 1971), whereas in non-ruminant animals, acetate is mainly metabolized in the liver and used for lipogenesis (Bauman and Davis, 1975).

The mechanism of nutrient absorption in the small intestine is different between ruminant and non-ruminant animals due to differences in the adaptive responses. For instance, pancreatic secretion of α-amylase is the limiting step for intestinal starch digestion in ruminant animals (Huntington, 1997) because of a lack of adaptive responses of the ruminant pancreas to increased dietary

starch intake (Kreikemeier et al., 1991), whereas in non-ruminant animals, pancreatic α-amylase secretion increases with high carbohydrate intake (Dagorn and Lahaie, 1981). Similarly, SGLT1 activity in ruminant intestinal mucosal cells is lower compared to that in non-ruminant animals (Bauer et al., 2001; Rodriguez et al., 2004).

2.1.2 Energy supply in ruminants

An adequate supply of energy is critical during peak lactation and advanced pregnancy. Negative energy balance is associated with metabolic and reproductive problems, and reduces milk yield in dairy cows (Baumgard et al., 2007; Jenkins and Ferrell, 1992). The milk energy output ranges from 11.l to 141.2 MJ/d, and there could be up to 88.2 MJ/d negative energy balance during lactation (Yan et al., 1997). Data calculated from primiparous cows showed that mean energy deficit per lactation was 776.8 MJ of NE_L and it took 41.47 days to return to positive energy balance (de Vries and Veerkamp, 2000). During the last two months of gestation, the energy requirements of a mature dairy cow is 30% higher than that of a non pregnant cow (Schingoethe et al., 1988).

Starch digested and absorbed as glucose produces more ATP compared to that obtained from digested and absorbed as acetate. Therefore, it is possible to improve energy supply in ruminants by enhancing intestinal glucose absorption (Owens et al., 1986).

2.1.3 Glucose supply in ruminants

Glucose is one of the limiting nutrients for high producing ruminant animals (Fahey and Gerger, 1988). The glucose requirement of ruminant animals is generally met by gluconeogenesis in the liver and kidney. However, previous reports indicated that glucose supply from gluconeogenesis alone may not be sufficient to meet the high glucose demand especially during peak lactation in high producing dairy cows (Elliot, 1976; Hurtaud et al., 2000). In dairy cows producing more than 30 kg milk per day, the glucose requirement in the mammary gland is equivalent to almost all of the glucose available in the body (Elliot, 1976). Increased supply of glucogenic nutrients increases milk yield (Lemosquet et al., 2004), indicating that glucose supply is limiting milk production in dairy cows.

2.1.4 Splanchnic metabolism

Splanchnic metabolism plays a central role in the delivery and utilization of nutrients (Seal and Parker, 2000). The splanchnic bed includes the GI tract, liver, spleen, pancreas, mesenteric fat, other connective tissues and blood vessels. It contributes approximately 15 - 20% of the total body mass in ruminant animals, and consumes 35 - 60% of the total oxygen in the body (Seal and Parker, 2000). Gut alone, which is about 6% of the body weight, consumes about 20 - 25% of the total oxygen (Britton and Krehbiel, 1993; Cant et al., 1996). The splanchnic metabolic activities in ruminant animals, therefore, are significant on a per unit mass basis.

Altered nutritional and physiological conditions can affect splanchnic metabolism. For instance, the relative proportion of nutrients in a diet affects energy, protein and carbohydrate metabolism by splanchnic tissues which can alter the amount of energy, glucose, amino-N and other nutrients available for production purposes (Huntington, 1989; Reynolds et al., 1991). Greater ammonia concentration in the mesenteric vein was reported to increase oxygen consumption in the splanchnic bed (Recavarren et al., 2004), and the net portal flux of ammonia increased with greater supply of metabolizable protein (MP) and rumen degradable protein (RDP; Blouin et al., 2002; Raggio et al., 2004). In addition, the absorption of amino acids in the portal vein increased when the energy supply was adequate to support rumen microbial protein synthesis (Reynolds et al., 1992). Further, heat stress increased the absorption of acetate and propionate, and oxygen uptake by the splanchnic tissues in sheep (Taniguchi et al., 2004).

2.2 Starch digestion in ruminant small intestine

Dietary starch is extensively fermented in the reticulo-rumen, and little starch reaches the small intestine for ruminant animals fed a high forage diet (Merchen, 1988). However, in the case of ruminant animals fed a high grain diet, approximately 44% of the starch consumed escapes ruminal fermentation and is available for intestinal digestion (Huntington, 1997; Owens et al., 1986). In the duodenal lumen, pancreatic α-amylase digests starch to maltose and

dextrin. Thereafter, the digestion takes place at the brush border membrane by the action of carbohydrases such as maltase and isomaltase. In ruminant animals, however, the activity of pancreatic α-amylase, and the brush border carbohydrases is probably limited due to the lack of adaptive response (Janes et al., 1985; Kreikemeier et al., 1991). It was observed that out of the total starch reaching the duodenum, 17 to 96% (Streeter et al., 1989; Zinn, 1991) was digested to glucose in the small intestine, but surprisingly approximately 45% of the starch entering the intestine was not absorbed as glucose (Huntington, 1997) probably due to the failure of starch digestion enzymes to respond to high starch concentration in the duodenum. Greater α-amylase activity was observed in increased dietary energy resulted from high starch diet (Kreikemeier et al., 1990) and increased duodenal protein supply (Huntington, 1997) in ruminants. Likewise, age and the physiological stage of the animals may also affect its activity (Kreikemeier et al., 1990; Swanson et al., 2002). Undigested starch is probably fermented in the large intestine or excreted, resulting in net loss of the starch consumed. Even though many studies showed that starch is available for post ruminal digestion, limitations to intestinal starch digestion for high yielding dairy cows especially in peak lactation are not known. The effect on the activity of pancreatic α-amylase and brush border carbohydrases in varying substrates concentrations in the duodenal lumen warrants further investigation.

2.3 Glucose metabolism in the small intestine

2.3.1 Glucose availability

The glucose availability and concentration in the small intestine is highly dependent on the diet (Harmon and McLeod, 2001). In ruminant animals fed a high forage diet, very little glucose is available in the intestinal lumen. Glucose from digestion of microbial polysaccharides (MacRae and Armstrong, 1969) and from intestinal mucin (Mukkur et al., 1985) is the only source of glucose in the intestinal lumen. Hepatic gluconeogenesis is the source of glucose required for metabolism in the intestinal tissues. In ruminant animals, the blood glucose concentration is 3 to 4 m*M* (Fernandez et al., 2001; Radosttitis et al., 1994). For ruminant animals fed a grain-based diet, it was observed that up to 44% of the dietary starch entered into the duodenum (Huntington, 1997; Owens et al., 1986), 17 to 96% of which was digested to glucose in the small intestine (Streeter et al., 1989; Zinn, 1991). In sheep fed 200 g hay and 700 g rolled barley, Heald (1951) observed that 16.8 - 21.9 g of α-linked glucose polymer entered into the small intestine per day. Glucose absorbed from the intestine can contribute up to 30% of the total glucose supply for cows fed a high concentrate diet, and therefore can be a significant glucose source to meet the glucose demand in lactating dairy cows. It was also observed that enhanced intestinal glucose supply increased mammary utilization of glucose and milk synthesis (Rigout et al., 2002), decreased hepatic gluconeogenesis (Clark et al., 1977), and increased oxidation of glucose by many tissues in the

body (Bartley and Black, 1966). Ruminant animals utilize the intestinal glucose more efficiently for the production of energy (Owens et al., 1986) and milk (Nocek and Tamminga, 1991) compared to the short chain VFA.

2.3.2 Glucose absorption in the intestinal mucosa

Insufficient absorption of glucose from the small intestine is one of the limitations of escape starch utilization in ruminants (Huntington et al., 2006). Glucose absorption capacity of the small intestine from adult ruminant animals is only 25% compared to that from the suckling pre-ruminant animals (White et al., 1971). Glucose transport across the intestinal brush border membrane is primarily via SGLT1 (Hediger and Rhoads, 1994). However, SGLT1 activity in the intestinal mucosa of cattle does not respond to a greater post ruminal starch concentration (Bauer et al., 2001; Rodriguez et al., 2004). However, increased glucose absorption was observed with higher glucose infusion in to the abomasum (Kreikemeier et al., 1991). This observation indicates that other transport mechanisms besides SGLT1 might be responsible for glucose absorption in cattle (Au et al., 2002). Another glucose transporter, GLUT2 is present in the basolateral membrane (Thorens et al., 1990) and apical membrane (Corpe et al., 1996) of the enterocytes. It contributes entry and exit of glucose from the enterocytes. GLUT2 has the capacity to respond rapidly to match the intestinal luminal glucose concentration (Kellett and Brot-Laroche, 2005). Paracellular diffusion, another mode of glucose absorption, contributes

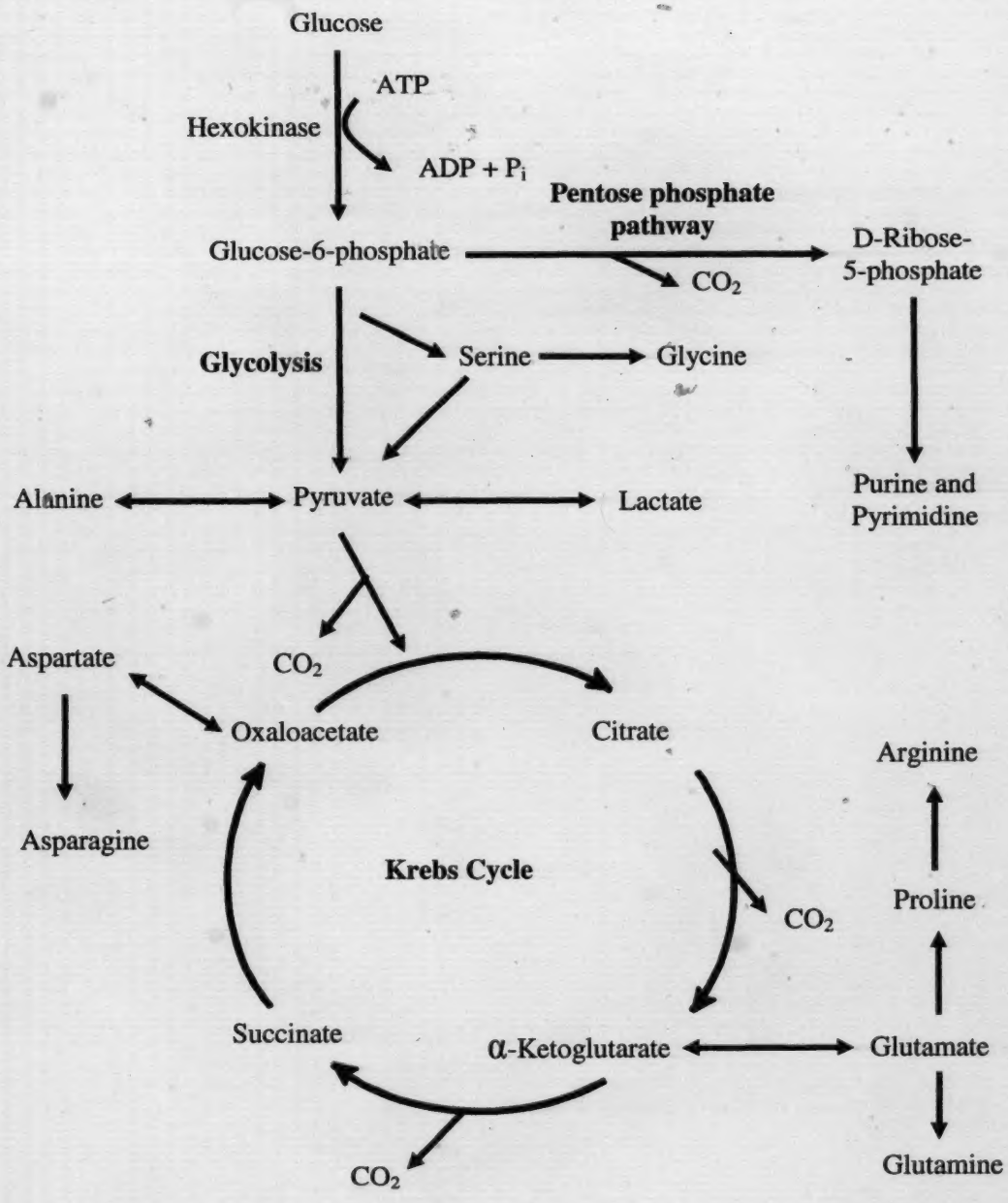
< 10% of the total glucose absorption in the intestine (Harmon and McLeod, 2001; Shirazi-Beechey et al., 1991).

Though previous reports provide valuable information about intestinal glucose absorption, there is still a gap in the literature regarding the regulatory mechanism of glucose transporters in the intestinal mucosa of lactating dairy cows fed a high starch diet.

2.3.3 Glucose metabolism pathways in DMC

The gut tissues utilize glucose by various metabolic pathways such as glycolysis, Krebs cycle, and pentose phosphate pathways. Wahle et al. (1971) observed that the ruminant gut tissues consumed glucose and produced lactate as one of the metabolic products. In a study using enterocytes isolated from lactating dairy cows, glucose was metabolized primarily to lactate, and 14 to 19% of the total metabolized glucose carbons appeared as CO_2 (Okine et al., 1995). The rate of CO_2 production from glucose increased with increasing glucose concentration in the incubation media, and glucose was more important than glutamine as an energy source for dairy cow enterocytes (Okine et al., 1995). In the pig, 14% of the glucose taken up by the enterocytes was metabolized via pentose phosphate pathway, and partial oxidation of glucose via this pathway dominated over Krebs cycle metabolism (Wu, 1996). In isolated ovine DMC, Oba et al. (2004a) observed that oxidative metabolism of glucose was flexible depending on the type and concentration of other

additional substrates such as propionate, glutamine and glutamate. The oxidation of glucose to CO_2 decreased in the presence of propionate, butyrate, glutamate and glutamine, but not in the presence of acetate. Furthermore, addition of glucose to the incubation media of isolated DMC increased the assimilation of nitrogen into alanine by 100%. This indicates that glucose might be a useful substrate for ammonia-N utilization in ruminant duodenal mucosa.



(Zubay et al., 1995)

Figure 2.1 Major possible pathways for glucose metabolism in the gut tissues

2.3.4 Effects of CP on glucose metabolism by intestinal mucosa

Previous in vivo studies (Richards et al., 2002; Taniguchi et al., 1995; Veira et al., 1980) indicated that abomasal or duodenal protein supply increased the net glucose absorption across the PDV when the amount of starch reaching the duodenum was high. In a steer receiving 800 g corn starch by abomasal infusion, supply of 800 g sodium caseinate in the abomasum increased the net glucose absorption across the PDV by 2 fold compared to the ruminal supply of the same quantity of sodium caseinate (Taniguchi et al., 1995). In weaned calves fed high-concentrate diets with 0 to 15% soybean meal replacing cracked corn, intestinal starch disappearance increased with increased dietary protein (Veira et al., 1980). Similarly in sheep, starch disappearance from the small intestine and glucose absorption in the portal vein increased with greater casein infusion in the abomasum (Castlebury and Preston, 1993). The reasons for the increased glucose absorption with greater CP supply in these studies could be due to increased activity of SGLT1 in greater concentrations of amino acids and peptides in the duodenum (Mabjeesh et al., 2003), and/or to the increased synthesis and secretion of pancreatic α-amylase from greater protein supply to the duodenum (Swanson et al., 2002).

Other studies (Blouin et al., 2002; El-Kadi et al., 2006; Guerino et al., 1991), however, reported that increased CP supply had either no or negative effects on net glucose absorption into the portal vein. Increased casein infusion into the duodenum (up to 105 g/day) had no effect on the amount of glucose

absorbed from the arterial circulation by the mesenteric drained viscera (MDV) in sheep fed a low starch (3.9%) diet (El-Kadi et al., 2006). In another study with dairy cows, increased MP intake (1930 vs. 1654 g/day) had no effect on net absorption of glucose into the portal vein (Blouin et al., 2002). Accordingly, in a study with steers fed a high grain (78% corn) diet, abomasal infusion of casein (150 g and 300 g /day) decreased the amount of glucose absorbed into the portal vein (Guerino et al., 1991).

2.3.5 Activity of enzymes involved in glucose metabolism

The first step in glucose metabolism is phosphorylation of glucose to glucose-6-phosphate, catalyzed by hexokinase. In enterocytes that have high turnover rate, high level of glucose-6-phophate is maintained for ribose synthesis (Newsholme et al., 1985). Hexokinase, therefore, is one of the most important enzymes involved in glucose metabolism in the enterocytes.

Hexokinase activity depends on various factors: animal and tissue types, presence of other metabolites, and pH. Hexokinase activity was 2.25 mU/mg protein in the chicken liver (Berradi et al., 2005) and up to 9 mU/mg protein in the liver from rats infused with insulin (Lam et al., 2003). The pH optimum for the activity of hexokinase is 7.5, and hexokinase rapidly loses its activity below pH 6.5 and above pH 8.5 with no enzymatic activity below pH 4.0 (Kumar et al., 2004). Hexokinase activity in rat intestinal mucosa was increased after a high-carbohydrate low-fat diet compared to a low-carbohydrate high-fat diet

(Srivastava et al., 1968). In a study with pancreatic islets, Liang et al. (1990) reported that activity of hexokinse increased by 236% when glucose concentration was increased from 5 to 30 m*M* in the incubation media. Likewise, hexokinase activity was increased in the presence of sucrose (Maiztegui et al., 2006), nitric oxide (Paik et al., 2005), phosphate ion, 3-phosphoglycerate, malate, citrate, ATP (Kosow and Rose, 1971), and protein (Stern, 1954) in the incubation media.

Hexokinase activity was found to be decreased in the presence of disulphides (Nesbakken and Eldjarn, 1963), progesterone (Sutter-Dub and Vergnaud, 1982), nerve and placenta extract, and plasma (Stern, 1954) in the incubation media. In addition, hypophyseal hormones may control hexokinase activity in rat small intestinal mucosa (Havivi and Levitan, 1968).

2.4 Volatile fatty acids metabolism in the gut tissues

Volatile fatty acids are produced as a microbial fermentation product in the GI tract of ruminants. The concentrations of VFA in the rumen are highly variable, although the total amount present usually is 60 - 150 m*M* (Bergman, 1990). Depending upon the diet composition and time after feeding, the proportion of acetate, propionate and butyrate in the total rumen VFA pool is 46 - 70, 19 - 42 and 11 - 13%, whereas, in the hindgut the ratio is approximately 70 : 20 :

10. Valeric acid and other mid-chain fatty acids constitute ≤ 5% of the total fermentation acids produced (Bergman, 1990).

The VFA can be absorbed across the rumen epithelium in both undissociated and dissociated forms. The undissociated VFA are lipophilic and diffuse across the epithelial membrane more easily than the dissociated form. The dissociated VFA are absorbed via anion exchange with bicarbonate (Britton and Krehbiel, 1993). In the rumen, the rate of VFA absorption increases with increasing chain length of the acid (Stevens and Stettler, 1966) due to greater lipid solubility. It was calculated that about 88% of the rumen VFA were directly absorbed from the rumen and only about 12% flowed to the omasum (Bergman, 1990).

Butyrate is preferentially metabolized in the rumen epithelium (Britton and Krehbiel, 1993) over acetate and propionate (Kristensen et al., 2000). About 90% of the total butyrate is metabolized by the rumen epithelium and formation of ketone bodies or oxidation to CO_2, accounts for most of the butyrate metabolism (Bergman, 1990). Previous studies reported that peripheral tissues utilize about 80% of total acetate in the blood (Bergman, 1990), while the PDV utilize up to 20% of available acetate (Annison and Armstrong, 1970) in ruminants. Acetate utilized by the small intestine is absorbed primarily from the arterial side (Seal and Parker, 1994). In isolated ovine DMC, the presence of acetate did not affect the oxidation of glucose,

glutamate, or glutamine, whereas, propionate reduced the oxidation of glucose and glutamate at high concentration (Oba et al., 2004a). In agreement, higher concentrations of propionate in the small intestine increased the net absorption of glutamine and glutamate across the MDV (Seal and Parker, 1996).

2.5 Nitrogen metabolism in the gut tissues

Rumen microbes break down dietary protein to amino acids, NH_3, VFA and CO_2, and synthesize both essential (EAA) and non-essential amino acids (NEAA). Depending upon dietary CP source, 60 to 90% of the total N intake by the ruminant is converted to ammonia (Mathison and Milligan, 1971). Excess ammonia and VFA are partly absorbed through the ruminal wall or enter into the small intestine. In the cow, of the total nitrogen available in the duodenum, EAA represented 35%, NEAA 30%, amides 4%, nucleic acids 11%, ammonia 6% and unknown 14% (Oldham and Tamminga, 1980). However, depending upon feeding management, dry matter intake, and dietary concentration of energy and CP, this proportion may vary greatly (Clark et al., 1992). It was also reported that the catabolism of amino acids is quantitatively greater than amino acid incorporation into the mucosal protein in the duodenum (Stoll et al., 1998).

2.5.1 Glutamine

Glutamine plays an important role in the metabolism of ruminant gut tissues (Heitmann and Bergman, 1978). The small intestine is the major site for the metabolism of circulating glutamine (Meister, 1984). Luminal glutamine is mainly transported by the sodium-dependent transport system with very little contribution from passive diffusion (Souba et al., 1992). Glutamine is mainly metabolized to ammonia, alanine, aspartate, glutamate, and CO_2 (Okine et al., 1995). In enterocytes, glutamine provides carbon and nitrogen precursors for various biosynthetic processes including citrulline synthesis (Okine et al., 1995; Wu et al., 1994). Glutamine produces energy via partial oxidation, and is one of the major fuels for enterocytes. Glutamine also stimulates the uptake of glucose, water and sodium ion by the intestine. However, glutamine is not quantitatively as important as glucose as an energy source in enteryocytes of lactating cows (Okine et al., 1995).

2.5.2 Glutamate

The small intestinal mucosa utilizes most of the glutamate (> 95%) present in the lumen (Reeds et al., 2000); however, intestinal uptake of arterial glutamate is not significant (Windmueller and Spaeth, 1975). In the pig, out of the total glutamate carbon from dietary sources, 63% appeared as portal CO_2, lactate and alanine, and about 10% was utilized for mucosal protein synthesis (Reeds et al., 2000). In isolated ruminant DMC, glutamate oxidation to CO_2 decreased in the presence of glutamine and propionate but not acetate, butyrate and

glucose (Oba et al., 2004a), indicating that ruminant enterocytes might have specific needs for glutamate oxidation that are not spared by the other substrates. Glutamate also acts as a carbon and nitrogen donor in the biosynthesis of proline, citrulline, arginine and glutathione (Reeds et al., 2000; Wu et al., 1994).

2.5.3 Other non-essential amino acids

The small intestinal mucosa metabolizes other NEAA, such as aspartate, alanine, leucine, serine and glycine. Luminal aspartate is extensively catabolized by the intestinal mucosa; however, arterial aspartate uptake is not significant (Windmueller and Spaeth, 1976; Wu, 1998). In the small intestine of rats, 50% of the metabolized aspartate carbon was recovered in CO_2, 24% in organic acids mostly lactate, 12% in alanine, glutamate, proline, ornithine and citrulline, and 10% in glucose. However, there was little or no production of ammonia from aspartate in the small intestine suggesting a dominant role of transamination in aspartate catabolism (Windmueller and Spaeth, 1976).

Alanine is synthesized from catabolism of glutamine, glutamate, and aspartate in the gut mucosa (Windmueller, 1982). Besides, ruminant DMC are capable of assimilating ammonia-N into amino-N by the synthesis of alanine (Oba et al., 2005), which can be a metabolic pathway to reduce the absorption of ammonia because alanine is net available across the PDV (El-Kadi et al., 2006), whereas other amino acids are net catabolized by the small intestinal

mucosa (Wu, 1998). It was also observed that ammonia-N assimilation into alanine by ovine DMC increased in the presence of glucose but not glutamate additions (Oba et al., 2005).

Leucine is also metabolized in the intestinal mucosa. Leucine is mainly used for protein synthesis and its catabolism is limited in the ovine small intestine (Cappelli et al., 1997). Leucine absorption across the PDV was not altered by the infusion of ammonium chloride into the mesenteric vein in sheep (Lobley et al., 1995). Besides, there are few reports about the metabolism of serine and glycine. In swine, 40 and 50% of dietary serine and glycine, respectively, were extracted in the first pass by the PDV, and < 20% of the extracted amino acids were utilized for intestinal protein synthesis (Stoll et al., 1998).

2.5.4 Essential amino acids

Small intestinal mucosa is capable of metabolizing EAA as well. About 50% of dietary lysine and methionine, 45% of dietary phenylalanine and 60% of dietary threonine were extracted in the first pass by swine PDV, and < 20% of the extracted EAA was utilized for mucosal protein synthesis (Stoll et al., 1998). It was observed that intestinal arginine catabolism is markedly increased in weaned pigs compared to new born piglets (Wu et al., 1996). Further, arginine can be a precursor for the biosynthesis of urea in intestinal mucosa (Wu, 1995).

2.5.5 Ammonia

In ruminants, about 60 to 90% of the total N intake is converted to ammonia in the GI tract (Mathison and Milligan, 1971) and is metabolized by various pathways. Depending upon the amount and source of dietary CP, the ammonia concentration in the ruminant GI tract varies greatly. Parker et al. (1995) reported that the basal ammonia level in the rumen was 2-4 m*M* and that it increased to 18-20 m*M* within 1 hr after silage feeding. Ammonia not used by the rumen microbes is readily absorbed across the gut epithelium mainly by diffusion due to its lipophilic nature (Huntington and Archibeque, 1999), whereas, ammonium ion is absorbed through an active transport mechanism (Mackie and White, 1990). In cattle, the intestinal absorption of ammonia accounted for up to 51% of net uptake of ammonia into the portal vein (Parker et al., 1995). Winnicka et al. (1992) reported that the normal blood ammonia concentration in sheep was reported to be about 0.1 m*M* (Winnicka et al., 1992).

Ammonia can be detoxified into urea or NEAA in the gut tissues. Enterocytes of pigs (Wu, 1995) and sheep (Oba et al., 2004b) are capable of converting ammonia-N into urea. The urea can later be recycled and used as a source of nitrogen in the rumen. Similarly, ruminant gut mucosa assimilates ammonia into NEAA such as: alanine, aspartate and glutamate (Oba et al., 2005). Ammonia combines with α-ketoglutarate to produce glutamate, which undergoes transamination reaction to produce other NEAA. Alanine synthesis

can be a useful metabolic pathway because it is net absorbed across the PDV (El-Kadi et al., 2006; Wu, 1998). Enhanced ammonia-N assimilation into alanine reduces net absorption of ammonia and it decreases the hepatic catabolism of other amino acids by providing additional amino-N for urea synthesis (Lobley et al., 1995).

Besides NEAA production, ruminant DMC can utilize ammonia-N to synthesize citrulline (Oba et al., 2004b) which is later converted to arginine in the kidney (Brosnan and Brosnan, 2004). However, due to limited or no activity of argino-succinate synthetase of the ornithine-urea cycle, ammonia transfer to urea is limited in the intestinal mucosa (Wu et al., 1994). Citrulline synthesis, therefore, does not increase with increased ammonia concentration (Mouillé et al., 1999). The absorption of amino-N from the intestine instead of ammonia-N is beneficial in two ways. Firstly, more amino-N is available to the animal and, secondly, the hepatic catabolism of amino acids for urea synthesis is reduced (Lobley et al., 1995) because less ammonia-N is present in the liver for detoxification.

2.6 Limitations of in vitro metabolism studies

In vitro experiments are useful to conduct metabolic studies in a controlled experimental setting. Isolated small intestinal mucosal cells have long been used for in vitro gut metabolism studies in rats (Ameh and Thompson, 1990;

Watford et al., 1978), chickens (Watford et al., 1978), guinea pigs (Gross et al., 1986) and swine (Wu, 1995). There are, however, very few studies (Oba et al., 2004a; Okine et al., 1995) using isolated DMC in ruminant animals. The isolation procedures used in these studies were different with respect to enzymes used to isolate cells, incubation method and buffer solution used.

The results of in vitro metabolism studies should be carefully interpreted due to some inherent limitations. In glucose metabolism studies in ruminant duodenal mucosa, the physiological milieu involved in glucose absorption and metabolism is different between the in vivo and in vitro conditions, For instances, glucose absorption is limited in ruminant duodenal mucosa in vivo (Huntington et al., 2006) probably because of a failure of SGLT1 to respond to higher luminal glucose concentrations in cattle (Bauer et al., 2001; Rodriguez et al., 2004). However, in isolated cells, both apical and basolateral membranes of the DMC come into direct contact with the incubation media containing glucose providing more opportunity for glucose transporter 2 (GLUT2: present mainly in the basolateral membrane; Thorens et al., 1990) to transport glucose. The GLUT2 has the capacity to respond rapidly to match the intestinal luminal glucose concentration (Kellett and Brot-Laroche, 2005). Due to the absence of various metabolites and hormones in the incubation media, isolated DMC metabolism might be different than the metabolism in DMC in vivo. Moreover, in vivo studies of ruminant gut tissues reported in the literature basically represent metabolism in the PDV, whereas in the isolated

DMC study, isolated mucosal epithelial cells are studied. The DMC represents a very small proportion of the PDV because the PDV contains varieties of tissues with diversified functions.

2.7 Conclusion

Productive and reproductive performance of high yielding dairy cows is negatively affected by a limited supply of glucose and energy during early lactation. Better understanding of nutrient metabolism in the GI tract is important to improve nutrient delivery to the peripheral tissues. Within the GI tract, the duodenal mucosa is one of the most active tissues involved in nutrient metabolism and absorption. Concentrations of nutrients and metabolites in the duodenum are highly variable depending on dietary and physiological factors. This variation can affect nutrient metabolism in the duodenal mucosa and their absorption across the PDV. For instance, altered pH, energy, and the presence of protein, VFA and NEAA can influence glucose metabolism in the DMC.

High dietary CP increases ammonia concentration in the GI tract and greater ammonia absorption negatively affects the nitrogen utilization efficiency in ruminant animals. However, ruminant DMC possess the metabolic capability to assimilate ammonia-N into NEAA and citrulline before the release into the portal vein. Alanine synthesis is a useful pathway to enhance greater net

systemic delivery of amino-N to the peripheral tissues. Ammonia-N incorporation into NEAA by DMC may increase in the presence of glucose. The regulatory mechanism of glucose carbon utilization for NEAA synthesis by the gut tissues and glucose absorption across the PDV at higher ammonia concentrations is yet to be studied.

2.8 References

Allen, R. S. 1982. Carbohydrate metabolism. Pages 327–335 in Duke's Physiology of Domestic Animals. 9th ed. M. J. Swenson, ed. Cornell Univ. Press, Ithaca, NY.

Ameh, D. A. and J. R. Thompson. 1990. Effect of *Escherichia coli* endotoxin on some aspects of amino acid and protein metabolism by the rat small intestine. Pages 1009–1016 in Amino Acids: Chemistry, Biology and Medicine. G. Lubec and G. A. Rosenthal, eds. Escom Science, Leiden, The Netherlands.

Annison, E. F. and D. G. Armstrong. 1970. Volatile fatty acid metabolism and energy supply. Pages 422–437 in Physiology of Digestion and Metabolism in the Ruminant. A. T. Phillipson, ed. Oriel, Newcastle upon Tyne, UK.

Au, A., A. Gupta, P. Schembri, and C. I. Cheeseman. 2002. Rapid insertion of GLUT2 into the rat jejunal brush-border membrane promoted by glucagon-like peptide 2. Biochem. J. 367:247–254.

Bartley, J. C. and A. L. Black. 1966. Effect of exogenous glucose on glucose metabolism in dairy cows. J. Nutr. 89:317–328.

Bauer, M. L., D. L. Harmon, D. W. Bohnert, A. F. Branco, and G. B. Huntington. 2001. Influence of alpha-linked glucose on sodium glucose cotransporter activity along the small intestine in cattle. J. Anim. Sci. 79:1917–1924.

Bauman, D. E. and C. L. Davis. 1975. Regulation of lipid metabolism. Page 496 in Digestion and Metabolism in the Ruminant. I. W. McDonald and A. C. I. Warner, eds. University of New England Publishing Unit, Armidale, Australia.

Baumgard, L. H., G. Schwartz, J. K. Kay, and M. L. Rhoads. 2007. Does negative energy balance limit milk synthesis in early lactation? Pages 77–87 in West. Canadian Dairy Seminar, Red Deer, AB.

Bergman, E. N. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol. Rev. 70:567–590.

Bergman, E. N., W. E. Roe, and K. Kon. 1966. Quantitative aspects of propionate metabolism and gluconeogenesis in sheep. Am. J. Physiol. 211:793–799.

Bergman, E. N. and J. E. Wolff. 1971. Metabolism of volatile fatty acids by liver and portal-drained viscera in sheep. Am. J. Physiol. 221:586–592.

Berradi, H., M. Taouis, S. Cassy, and N. Rideau. 2005. Glucokinase in chicken (Gallus gallus): Partial cDNA cloning, immunodetection and activity determination. Comp. Biochem. Physiol. B. 141:129–139.

Blouin, J. P., J. F. Bernier, C. K. Reynolds, G. E. Lobley, P. Dubreuil, and H. Lapierre. 2002. Effect of supply of metabolizable protein on splanchnic fluxes of nutrients and hormones in lactating dairy cows. J. Dairy Sci. 85:2618–2630.

Britton, R. and C. Krehbiel. 1993. Nutrient Metabolism by Gut Tissues. J. Dairy. Sci. 76:2125–2131.

Brosnan, M. E. and J. T. Brosnan. 2004. Renal arginine metabolism. J. Nutr. 134:2791S–2795S.

Cant, J. P., B. W. McBride, and W. J. Croom. 1996. The regulation of Intestinal Metabolism and its Impact on whole animal energetics. J. Anim. Sci. 74:2541–2553.

Cappelli, F. P., C. J. Seal, and D. S. Parker. 1997. Glucose and [^{13}C]leucine metabolism by the portal-drained viscera of sheep fed on dried grass with acute intravenous and intraduodenal infusions of glucose. Br. J. Nutr. 78:931–946.

Castlebury, R. E. and R. L. Preston. 1993. Effect of dietary protein level on nutrient digestion in lambs duodenally infused with cornstarch. J. Anim. Sci. 71(Suppl. 1):264. (Abstr.)

Clark, J. H., T. H. Klusmeyer, and M. R. Cameron. 1992. Microbial protein synthesis and flows of nitrogen fractions to the duodenum of dairy cows. J. Dairy. Sci. 75:2304–2323.

Clark, J. H., H. R. Spires, R. G. Derrig, and M. R. Bennink. 1977. Milk production, nitrogen utilization and glucose synthesis in lactating cows infused postruminally with sodium caseinate and glucose. J. Nutr. 107:631–644.

Corpe, C. P., M. M. Basaleh, J. Affleck, G. Gould, T. J. Jess, and G. L. Kellett. 1996. The regulation of GLUT5 and GLUT2 activity in the adaptation of intestinal brush-border fructose transport in diabetes. Pflugers Arch. Eur. J. Physiol. 432:192–201.

Dagorn, J. C. and R. G. Lahaie. 1981. Dietary regulation of pancreatic protein synthesis I: rapid and specific modulation of enzyme synthesis by changes in dietary composition. Biochim. Biphys. Acta. 654:111–118.

de Vries, M. J. and R. F. Veerkamp. 2000. Energy balance of dairy cattle in relation to milk production variables and fertility. J. Dairy Sci. 83:62–69.

El-Kadi, S. W., R. L. Bladwin VI, N. E. Sunny, S. L. Owens, and B. J. Bequette. 2006. Intestinal protein supply alters amino acid, but not glucose, metabolism by the sheep gastrointestinal tract. J. Nutr. 136:1261–1269.

Elliot, J. M. 1976. The glucose economy of the lactating dairy cow. Pages 59–66 in Proc. Cornell Nutr. Conf. Feed Manuf., Ithaca, NY.

Fahey Jr., G. C. and L. L. Gerger. 1988. Carbohydrate nutrition of ruminants. Prentice Hall, Englewood Cliffs, NJ.

Fernandez, J. M., T. Sahlu, S. P. Hart, M. J. Potchoiba, H. M. E. Shaer, N. Jacquemet, and H. Carneiro. 2001. Experimentally-induced subclinical hyperammonemia in dairy goats. Small Ruminant Research. 42:5–20.

Gross, C. J., L. M. Henderson, and D. A. Savaiano. 1986. Uptake of L-carnitine, D-carnitine and acetyl-L-carnitine by isolated guinea pig enterocytes. Biochim. Biophys. Acta. 886:425–433.

Guerino, F., G. B. Huntington, and R. A. Erdman. 1991. The net portal and hepatic flux of metabolites and oxygen consumption in growing beef steers given postruminal casein. J. Anim. Sci. 69:387–395.

Harmon, D. L. and K. R. McLeod. 2001. Glucose uptake and regulation by intestinal tissues: Implications and whole-body energetics. J. Anim. Sci. 79(E. Suppl.):E59–E72.

Havivi, E. and R. Levitan. 1968. Hexokinase activity in intestinal mucosa of hypophysectomized rats. Am. J. Physiol. 215:330–333.

Heald, P. J. 1951. The estimation of glucose containing substances in microorganisms from the rumen of the sheep. Br. J. Nutri. 9:75–83.

Hediger, M. A. and D. B. Rhoads. 1994. Molecular physiology of sodium glucose cotransporters. Physiol. Rev. 74:993–1026.

Heitmann, R. N. and E. N. Bergman. 1978. Glutamine metabolism, interorgan transport, and glucogenicity in the sheep. Am. J. Physiol. 234:E197–E203.

Huntington, G. B. 1989. Hepatic urea synthesis and site and rate of urea removal from blood of beef steers fed alfalfa hay or a high concentrate diet. Can. J. Anim. Sci. 69:215–223.

Huntington, G. B. 1997. Starch utilization by ruminants: From basics to the bunk. J. Anim. Sci. 75:852–867.

Huntington, G. B. and S. L. Archibeque. 1999. Practical aspects of urea and ammonia metabolism in ruminants. Proc. Am. Soc. Anim. Sci. 1999. Available: http://www.asas.org/jas/symposia/proceedings/0939.pdf. Accessed Jan. 15, 2007.

Huntington, G. B., D. L. Harmon, and C. J. Richards. 2006. Sites, rates, and limits of starch digestion and glucose metabolism in growing cattle. J. Anim. Sci. 84 (E. Suppl.):E14–E24.

Hurtaud, C., S. Lemosquet, and H. Rulquin. 2000. Effect of graded duodenal infusions of glucose on yield and composition of milk from dairy cows. 2. Diets based on grass silage. J. Dairy Sci. 83:2952–2962.

Janes, A. N., T. E. C. Weekes, and D. G. Armstrong. 1985. Carbohydrase activity in the pancreatic tissue and small intestine mucosa of sheep fed dried-grass or ground maize-based diets. J. Agric. Sci. (Camb.). 104:435–443.

Jenkins, T. G. and C. L. Ferrell. 1992. Lactation characteristics of nine breeds of cattle fed various quantities of dietary energy. J. Anim. Sci. 70:1652–1660.

Kellett, G. L. and E. Brot-Laroche. 2005. Apical GLUT2: A major pathway of intestinal sugar absorption. Diabetes. 54:3056–3062.

Kosow, D. P. and I. A. Rose. 1971. Activators of yeast hexokinase. J. Biol. Chem. 246:2618–2625.

Kreikemeier, K. K., D. L. Harmon, J. P. Peters, K. L. Gross, C. K. Armendariz, and C. R. Krehbiel. 1990. Influence of dietary forage and feed intake on carbohydrase activities and small intestinal morphology of calves. J. Anim. Sci. 68:2916–2929.

Kreikemeier, K. K., D. L. Harmon, J. R. T. Brandt, T. B. Avery, and D. E. Johnson. 1991. Small intestinal starch digestion in steers: Effect of

various levels of abomasal glucose, corn starch and corn dextrin infusion on small intestinal disappearance and net glucose absorption. J. Anim. Sci. 69:328–338.

Kristensen, N. B., G. Gäbel, S. G. Pierzynowski, and A. Danfær. 2000. Portal recovery of short-chain fatty acids infused into the temporarily isolated and washed reticulo-rumen of sheep. Br. J. Nutr. 84:477–482.

Kumar, D. P., A. Tiwari, and R. Bhat. 2004. Effect of pH on the stability and structure of yeast hexokinase A: acidic amino acid residues in the cleft region are critical for the opening and the closing of the structure. J. Biol. Chem. 279:32093–32099.

Lam, T. K. T., G. Van de Werve, and A. Giacca. 2003. Free fatty acids increase basal hepatic glucose production and induce hepatic insulin resistance at different sites. Am. J. Physiol. Endocrinol. Metab. 284:e281–e290.

Lemosquet, S., S. Rigout, A. Bach, H. Rulquin, and J. W. Blum. 2004. Glucose metabolism in lactating cows in response to isoenergic infusions of propionic acid or duodenal glucose. J. Dairy Sci. 87:1767–1777.

Liang, Y., H. Najafi, and F. M. Matschinsky. 1990. glucose regulates glucokinase activity in cultured islets from rat pancreas. J. Biol. Chem. 265:16863–16866.

Lobley, G. E., A. Connell, M. A. Lomax, D. S. Brown, E. Milne, A. G. Calder, and D. A. Farningham. 1995. Hepatic detoxification of ammonia in the

ovine liver: possible consequences for amino acid catabolism. Br. J. Nutr. 73:667–685.

Mabjeesh, S. J., D. Guy, and D. Sklan. 2003. Na^+ glucose co-transporter abundance and activity in the small intestine of lambs: Enhancement by abomasal infusion of casein. Br. J. Nutr. 89:573–580.

Mackie, R. I. and B. A. White. 1990. Recent advances in rumen microbial ecology and metabolism: potential impact on nutrient output. J. Dairy Sci. 73:2971–2995.

MacRae, J. C. and D. G. Armstrong. 1969. Studies on intestinal digestion in the sheep. Br. J. Nutr. 23:377–387.

Maiztegui, B., M. I. Borelli, M. L. Massa, H. Del Zotto, and J. J. Gagliardino. 2006. Enhanced expression of hexokinase I in pancreatic islets induced by sucrose administration. J. Endocrinol. 189:311–317.

Mathison, G. W. and L. P. Milligan. 1971. Nitrogen metabolism in sheep. Br. J. Nutr. 25:351–366.

Meister, A. 1984. Enzymology of glutamine. Page 298 in Glutamine Metabolism in Mammalian tissues. D. Haussinger, and H. Sies, eds. Springer-Verlag, Berlin.

Merchen, N. R. 1988. Digestion, absorption and excretion in ruminants. Page 172 in The Ruminant Animal: Digestive Physiology and Nutrition. D. C. Church, ed. Prentice-Hall, Englewood Cliffs, NJ.

Mouillé, B., E. Moreal, V. Robert, G. Guihot-Joubrel, and F. Blachier. 1999. Metabolic capacity for citrulline synthesis from ammonia in rat isolated colonocytes. Biochim. Biophys. Acta. 1427:401–407.

Mukkur, T. K., D. L. Watson, K. S. Saini, and A. K. Lascelles. 1985. Purification and characterization of goblet–cell mucin of high M_r from the small intestine of sheep. Biochem. J. 229:419–428.

Nesbakken, R. and L. Eldjarn. 1963. The inhibition of hexokinase by disulphides. Biochem. J. 87:526–532.

Newsholme, E. A., B. Crabtree, and M. S. M. Ardawi. 1985. The role of high rates of glycolysis and glutamine utilization in rapidly dividing cells. Biosci. Rep. 5:393–400.

Nocek, J. E. and S. Tamminga. 1991. Site of digestion of starch in the gastrointestinal tract of dairy cows and Its effect on milk yield and composition. J. Dairy Sci. 74:3598–3629.

Oba, M., R. L. Baldwin VI, and B. J. Bequette. 2004a. Oxidation of glucose, glutamate, and glutamine by isolated ovine enterocytes in vitro is decreased by the presence of other metabolic fuels. J. Anim. Sci. 82:479–486.

Oba, M., R. L. Baldwin VI, S. L. Owens, and B. J. Bequette. 2004b. Urea synthesis in ruminal epithelial and duodenal mucosal cells isolated from growing sheep. J. Dairy Sci. 87:1803–1805.

Oba, M., R. L. Baldwin VI, S. L. Owens, and B. J. Bequette. 2005. Metabolic fates of ammonia in ruminal epithelial and duodenal mucosal cells isolated from growing sheep. J. Dairy Sci. 88:3963–3970.

Okine, E. K., D. R. Glimm, J. R. Thompson, and J. J. Kennelly. 1995. Influence of stage of lactation on glucose and glutamine metabolism in isolated enterocytes from dairy cattle. Metabolism. 44:325–331.

Oldham, J. D. and S. Tamminga. 1980. Amino acid utilization by dairy cows. I. Methods of varying amino acid supply. Livest. Prod. Sci. 7:437–452.

Owens, F. N., R. A. Zinn, and Y. K. Kim. 1986. Limits to starch digestion in the ruminant small intestine. J. Anim. Sci. 63:1634–1648.

Paik, J. Y., K. H. Lee, B. H. Ko, Y. S. Choe, Y. Choi, and B. T. Kim. 2005. Nitric oxide stimulates 18F-FDG uptake in human endothelial cells through increased hexokinase activity and GLUT1 expression. J. Nuclear Med. 46:365–370.

Parker, D. S., M. A. Lomax, C. J. Seal, and J. C. Wilton. 1995. Metabolic implications of ammonia production in the ruminants. Proc. Nutr. Soc. 54:549–563.

Radosttitis, O. M., D. C. Blood, and C. C. Gay. 1994. Veterinary Medicine: A Textbook of the Diseases of Cattle, Sheep, Pigs, Goats and Horses. 8th ed. ELBS. Bailliere Tindall, London.

Raggio, G., D. Pacheco, R. Berthiaume, G. E. Lobley, D. Pellerin, G. Allard, P. Dubreuil, and H. Lapierre. 2004. Effect of level of metabolizable protein

on splanchnic flux of amino acids in lactating dairy cows. J. Dairy Sci. 87:3461–3472.

Recavarren, M. I., M. J. D. Sole, and G. D. Milano. 2004. Splanchnic net balance of oxygen and metabolites in response to mesenteric vein infusion of ammonia in sheep. J. Anim. Feed. Sci. 13(Suppl. 1):335–338.

Reeds, P. J., D. G. Burrin, B. Stoll, and F. Jahoor. 2000. Intestinal Glutamate Metabolism. J. Nutr. 130:978S–982S.

Reynolds, C. K., P. C. Aikman, B. Lupoli, D. J. Humphries, and D. E. Beever. 2003. Splanchnic metabolism of dairy cows during the transition from late gestation through early gestation. J. Dairy Sci. 86:1201–1217.

Reynolds, C. K., D. P. Casper, D. L. Harmon, and C. T. Milton. 1992. Effect of CP and ME intake on visceral nutrient metabolism in beef steers. J. Anim. Sci. 70(Suppl. 1):315. (Abstr.).

Reynolds, C. K., G. B. Huntington, H. F. Tyrrell, and P. J. Reynolds. 1988. Net portal-drained visceral and hepatic metabolism of glucose, L-lactate, and nitrogenous compounds in lactating Holstein cows. J. Dairy Sci. 71:1803–1812.

Reynolds, C. K., H. F. Tyrrell, and P. J. Reynolds. 1991. Effect of diet forage-to-concentrate ratio and intake on energy metabolism in growing heifers: net nutrient metabolism by visceral tissues. J. Nutr. 121:1004–1015.

Richards, C. J., A. F. Branco, D. W. Bohnert, G. B. Huntington, M. Macari, and D. L. Harmon. 2002. Intestinal starch disappearance increased in steers abomasally infused with starch and protein. J. Anim. Sci. 2080:3361–3368.

Rigout, S., S. Lemosquet, J. E. Van Eys, J. W. Blum, and H. Rulquin. 2002. Duodenal glucose increases glucose fluxes and lactose synthesis in grass silage-fed dairy cows. J. Dairy Sci. 85:595–606.

Rodriguez, S. M., K. C. Guimaraes, J. C. Matthews, K. R. McLeod, R. L. Baldwin, and D. L. Harmon. 2004. Influence of abomasal carbohydrates on small intestinal sodium-dependent glucose cotransporter activity and abundance in steers. J. Anim. Sci. 82:3015–3023.

Schingoethe, D. J., F. M. Byers, and G. T. Schelling. 1988. Nutrient needs during periods of the life cycle. Pages 269–297 in The Ruminant Animal: Digestive Physiology and Nutrition. D. C. Church, ed. Prentice-Hall, Englewood Cliffs, NJ.

Seal, C. J. and D. S. Parker. 1994. Effect of intraruminal propionic acid infusion on metabolism of mesenteric- and portal-drained viscera in growing steers fed a forage diet: I. Volatile fatty acids, glucose, and lactate. J. Anim. Sci. 72:1325–1334.

Seal, C. J. and D. S. Parker. 1996. Effect of intraruminal propionic acid infusion on metabolism of mesenteric and portal drained viscera in growing steers fed a forage diet: II. Ammonia, urea, amino acids, and peptides. J. Anim. Sci. 74:245–256.

Seal, C. J. and D. S. Parker. 2000. Influence of gastrointestinal metabolism on substrate supply to the liver. Page 474 in Ruminant Physiology: Digestion, Metabolism, Growth and Reproduction. P. B. Cronje, ed. CAB International Publishing, Wallingford, UK.

Shirazi-Beechey, S. P., B. A. Hirayama, Y. Wang, D. Scott, M. W. Smith, and E. M. Wright. 1991. Ontogenic development of lamb intestinal sodium-glucose co-transporter is regulated by diet. J. Physiol. 437:699–708.

Souba, W. W., M. Pan, and B. R. Stevens. 1992. Kinetics of the sodium-dependent glutamine transporter in human intestinal cell confluent monolayers. Biochem. Biophys. Res. Commun. 188:746–753.

Srivastava, L. M., P. Shakespeare, and G. Hubscher. 1968. Glucose metabolism in the mucosa of the small intestine: a study of hexokinase activity. Biochem. J. 109:35–42.

Stern, J. 1954. Inhibitors and activators of brain hexokinase. Biochem. J. 58:536–542.

Stevens, C. E. and B. K. Stettler. 1966. Transport of fatty acid mixtures across rumen epithelium. Am. J. Physiol. 211:264–271.

Stoll, B., J. Henry, P. J. Reeds, H. Yu, F. Jahoor, and D. G. Burrin. 1998. Catabolism dominates the first-pass intestinal metabolism of dietary essential amino acids in milk protein fed piglets. J. Nutr. 128:606–614.

Streeter, M. N., D. G. Wagner, C. A. Hibberd, and F. N. Owens. 1989. Combinations of high-moisture harvested sorghum grain and dry-rolled

corn: Effects on site and extent of digestion in beef heifers. J. Anim. Sci. 67:1623–1633.

Sutter-Dub, M. T. and M. T. Vergnaud. 1982. Progesterone and glucose metabolism in the female rat adipocyte: effect on hexokinase activity. J. Endocrinol. 95:369–375.

Swanson, K. C., J. C. Matthews, C. A. Woods, and D. L. Harmon. 2002. Postruminal administration of partially hydrolyzed starch and casein influences pancreatic a-amylase expression in calves. J. Nutr. 132:376–381.

Taniguchi, K., G. B. Huntington, and B. P. Glenn. 1995. Net nutrient flux by visceral tissues of beef steers given abomasal and ruminal infusions of casein and starch. J. Anim. Sci. 73:236–249.

Taniguchi, K., K. Nakamura, T. Yoneyama, T. Ito, T. Sugino, and T. Obitsu. 2004. Effects of short-term exposure to heat stress on splanchnic metabolism in sheep. J. Anim. Feed Sci. 13 (Suppl. 1):359–362.

Thorens, B., Z. Q. Cheng, D. Brown, and H. F. Lodish. 1990. Liver glucose transporter: a basolateral protein in hepatocytes and intestine and kidney cells. Am. J. Physiol. Endocrinol. Metab. 259:C279-C285.

Veira, D. M., G. K. MacLoed, J. H. Burton, and J. B. Stone. 1980. Nutrition of the weaned Holstein calf. II. Effect of dietary protein level on nitrogen balance, digestibility and feed intake. J. Anim. Sci. 50:945–951.

Wahle, K. W. J., D. G. Armstrong, and H. S. A. Sherratt. 1971. The metabolism of the small intestine: Glycolysis in the mucosa of the small intestine of the sheep. Comp. Biochem. Physiol. B. 38:5-20. (Abstr.)

Watford, M., P. Lund, and H. A. Krebs. 1978. Isolation and metabolic characteristics of rat and chicken enterocytes. Biochem. J. 178:589–596.

Weigand, E., J. W. Young, and A. D. McGilliard. 1975. Volatile fatty acid metabolism by rumen mucosa from cattle fed hay or grain. J. Dairy Sci. 58:1294–1300.

White, R. G., V. J. Williams, and R. J. Morris. 1971. Acute in vivo studies on glucose absorption from the small intestine of lambs, sheep and rats. Br. J. Nutr. 25:57–76.

Windmueller, H. G. 1982. Glutamine utilization by the small intestine. Adv. Enzymol. 53:201–237.

Windmueller, H. G. and A. E. Spaeth. 1975. Intestinal metabolism of glutamine and glutamate from the lumen as compared to glutamine from blood. Arch. Biochem. Biophys. 171:662–672.

Windmueller, H. G. and A. E. Spaeth. 1976. Metabolism of absorbed aspartate, asparagine, and arginine by rat small intestine in vivo. Arch. Biochem. Biophys. 175:670–676.

Winnicka, A. W., K. P. Ostaszewski, E. Miernik-Degnrska, and A. Degnrski. 1992. Effect of subclinical hyperammonaemia on phagocytic activity of

polymorphonuclear leukocytes isolated from peripheral blood of sheep. Small Ruminant Research. 7:253–264.

Wu, G. 1995. Urea synthesis in enterocytes of developing pigs. Biochem. J. 312:717–723.

Wu, G. 1996. An important role of pentose cycle in the synthesis of citrulline and proline from glutamine in porcine enterocytes. Arch. Biochem. Biophys. 336:224–230.

Wu, G. 1998. Intestinal mucosal amino acid catabolism. J. Nutr. 128:1249–1252.

Wu, G., D. A. Knabe, and N. E. Flynn. 1994. Synthesis of citrulline from glutamine in pig enterocytes. Biochem. J. 299:115–121.

Wu, G., D. A. Knabe, N. E. Flynn, W. Yan, and S. P. Flynn. 1996. Arginine degradation in developing porcine enterocytes. Am. J. Physiol. 271:G913–G919.

Yan, T., F. J. Gordon, R. E. Agnew, M. G. Porter, and D. C. Patterson. 1997. The metabolisable energy requirement for maintenance and the efficiency of utilization of metabolisable energy for lactation by dairy cows offered grass silage-based diets. Livest. Prod. Sci. 51:141–150.

Zinn, R. A. 1991. Comparative feeding value of steam-flaked corn and sorghum in finishing diets supplemented with or without sodium bicarbonate. J. Anim. Sci. 69:905–916.

Zubay, G. L., W. W. Parson, and D. E. Vance. 1995. Principles of Biochemistry. 1st ed. Wm. C. Brown, Dubuque, IO.

CHAPTER 3

Evaluation of procedures for the isolation of ruminant enterocytes

3.1 Introduction

The gastrointestinal tract is one of the most active organs in nutrient metabolism (Britton and Krehbiel, 1993; Cant et al., 1996) and within the gastrointestinal tract, the duodenum is the primary site of nutrient digestion and absorption. Nutrient metabolism in the duodenal mucosal cells (DMC) can, therefore, have a significant impact on the availability of nutrients to peripheral tissues.

The metabolic capabilities of DMC in ruminant animals and their physiological basis have received significant attention recently. Isolated small intestinal mucosal cells have been used to address specific physiological questions in a controlled experimental setting in rats (Ameh and Thompson, 1990; Watford et al., 1978), chickens (Watford et al., 1978), guinea pigs (Gross et al., 1986) and swine (Wu, 1995). Intestinal mucosal cell isolation procedures have already been established in these species. There are, however, very few studies (Oba et al., 2004; Okine et al., 1995) using isolated DMC in ruminant animals, and the isolation procedures described by these authors have not been tested by others. Therefore, it is not yet known which procedure is better for ruminant DMC isolation. In addition, the procedure used to isolate jejunal mucosal cells in pigs (Wu, 1995) has not been evaluated for the isolation of ruminant DMC.

The major differences in the DMC isolation procedures among these studies (Oba et al., 2004; Okine et al., 1995; Wu, 1995) are the incubation method, and whether or not a tissue dissociating enzyme solution is used. The objective of the current study was to evaluate the efficacy of three previously reported small intestinal mucosal cell isolation procedures (Oba et al., 2004; Okine et al., 1995; Wu, 1995) for the isolation of ruminant DMC.

3.2. Materials and Methods

3.2.1 Collection of duodenal segment

The duodenal tissue specimens used for the study were obtained from steers slaughtered in a commercial abattoir (Edmonton Custom Packers, Edmonton, AB). From each donor steer, a section of the duodenum (50-cm long) was excised 30 cm distal to the pylorus immediately after slaughter and transported to the laboratory within 30 minutes for the isolation of DMC.

3.2.2 Duodenal mucosal cell isolation

The DMC isolation procedures previously used for sheep (procedure A; Oba et al., 2004), cows (procedure B; Okine et al., 1995), and the jejunal mucosal cell isolation procedure for pigs (procedure C; Wu, 1995) were used to isolate DMC from the excised duodenum. Each procedure was repeated four times.

3.2.2.1 Procedure A

The excised duodenum was rinsed with Krebs-Ringer buffer with HEPES (KRB-HEPES buffer: 120 m*M* NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 m*M* $NaHCO_3$, and 25 m*M* HEPES, oxygenated with O_2:CO_2 [95:5], pH 7.4) to remove feed particles and mucus, split longitudinally, and the mucosa scraped off longitudinally using a glass slide from the underlying musculature. The collected mucosa was minced and placed in a flask containing 250 mL digestion solution containing collagenase (300 units/mL; Worthington Biochemical Corporation, Lakewood, NJ), dispase (0.1 mg/mL; Worthington) and $CaCl_2$ (0.14 mg/mL). Dispase and collagenase, respectively, disintegrate the tight junction of mucosal epithelium and the attachment of the basement membrane to the submucosa (Martinez-Hernandez and Amenta, 1983; Tarkkanen et al., 1990). Scraped mucosal cells in the digestion solution were then incubated in a shaking water bath (Julabo SW 22, Germany) at 37°C for 20 minutes. The liberated mucosal cells were separated from mucosal remnants by sequential filtration through a 1000 µm and a 300 µm polypropylene mesh (Spectra/Mesh, Spectrum Laboratory Products, Los Angeles, CA) without vacuum. The filtrate containing enterocytes was centrifuged at 60 × *g* for six minutes (Centra-MP4R, International Equipment Company, Needham Heights, MA). The supernatant was discarded and the pellet was washed with KRB-HEPES buffer and centrifuged again at 60 × *g* for six minutes. The resulting pellet was suspended in KRB-HEPES buffer.

3.2.2.2 Procedure B

This procedure is a modification of a procedure used to isolate jejunal mucosal cells from rats (Ameh and Thompson, 1990). The excised duodenum was flushed with KRB-HEPES buffer to remove the digesta and mucus. The distal end of the tissue was ligated with a tubing clamp (Fisher Scientific Co., Ottawa, ON) and the segment was filled with freshly oxygenated KRB-HEPES buffer containing 2.5 mg/mL bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO). The proximal end was then ligated with another tubing clamp and the tissue was incubated in a container that contained KRB-HEPES buffer for 15 minutes in a shaking water bath at 37°C. The duodenal contents were discarded and the segment was again filled with the digestion solution; freshly oxygenated KRB-HEPES buffer that contained 2.5 mg/mL BSA and 1.5 mg/mL hyaluronidase (Worthington). The re-filled segments containing the digestion solution were kept on crushed ice in a container, and gently patted with fingertips for two minutes to release the DMC into the lumen. The resulting cells were washed three times with solution B after centrifugation at 1,000 × *g* (Centra-MP4R) for three minutes each time.

3.2.2.3 Procedure C

This procedure is also a modification of a technique to isolate jejunal mucosal cells for chicken and rat (Watford et al., 1978). The excised duodenum was obtained and rinsed with fresh cold phosphate buffer solution (PBS: 137 m*M* NaCl, 2.7 m*M* KCl, 1.4 m*M* KH_2PO_4, 4.3 m*M* Na_2HPO_4, pH 7.4) to remove

digesta and mucus. The section was rinsed three times with freshly oxygenated Ca^{2+}-Free Krebs-Henseleit bicarbonate buffer with HEPES (KHB-HEPES buffer: 119 m*M* NaCl, 4.8 m*M* KCl, 1.2 m*M* $MgSO_4$, 1.2 m*M* KH_2PO_4, 25 m*M* $NaHCO_3$, 20 m*M* HEPES, oxygenated with O_2:CO_2 [19:1] pH 7.4) containing 5 m*M* disodium EDTA. The tissue was filled with the KHB-HEPES buffer containing EDTA, closed with tubing clamps at both ends and completely immersed in a container containing the same buffer, and then incubated at 37°C in a shaking water bath (70 oscillations/min) for 50 minutes (Julabo SW 22). At the end of the incubation, the tissue was gently patted with fingertips for two minutes. The contents were filtered through two layers of cheesecloth and collected in a beaker. The filtered cells were then centrifuged at 400 × *g* for three minutes at 4°C (Centra-MP4R) and washed three times in KHB-HEPES buffer containing 2.5 m*M* $CaCl_2$ but no EDTA.

3.2.3 Viability determination and cell count

Viability was determined by the trypan blue exclusion test using 0.8 m*M* trypan blue dye in PBS (Kaltenbach, 1958) and the number of cells per mL was estimated using a hemocytometer. Dye exclusion test is the commonly used technique to determine the viability of ruminant gut epithelial cells (Baldwin and McLeod, 2000; Oba, 2004; Okine et al., 1995). Cell viability is determined from cell membrane integrity in this test. It is possible that cells' metabolic activity can be reduced even though its membrane integrity is maintained (Strober, 1997).

3.2.4 Statistical analysis

Data were analyzed using the fit Y by X procedure of JMP software (SAS Institute, Inc., Cary, NC) with the fixed effect of isolation procedures in the model. Treatment effects were declared significant at $P < 0.05$ and least significant difference was used to test the difference among the means.

3.3. Results and discussion

The total viable isolated cells from a 50-cm long excised duodenal segment were higher ($P < 0.05$) for the procedure B ($1.85 \times 10^9 \pm 0.04 \times 10^9$) compared to the procedures A ($1.66 \times 10^9 \pm 0.04 \times 10^{9)}$ and C ($1.58 \times 10^9 \pm 0.07 \times 10^9$; Figure 3.1). In procedure B, use of hyaluronidase along with shaking probably expedited the rate of DMC isolation compared to procedures A and C because of more efficient disintegration of cell-cell adhesive contacts (Underhill, 1989).

Cell viability immediately after isolation was significantly greater ($P < 0.05$) for the procedures A (85.5 ± 0.8%) and B (88.5 ± 1.7%) compared to procedure C (78.7 ± 1.8%; Figure 2). The viability of DMC immediately after isolation using procedure A and procedure B in this study, was similar to previous reports for sheep (Oba et al., 2004) and cows (Okine et al., 1995), respectively. The viability of the cells isolated by using procedure C was, however, lower than that reported for pigs (Wu, 1995). The discrepancy might be due to the

differences in animal species and/or location of the small intestine segment used (duodenum vs. jejunum).

The reduction in cell viability over 6 hours at room temperature after isolation was lower ($P < 0.05$) for procedure A (1.04 ± 0.07 %), compared with procedures B (2.52 ± 0.16 %) and C (3.73 ± 0.31 %). Since scraped duodenal cells were isolated using dispase and collagenase, procedure A might have also isolated cells from inner tissue layers particularly from plica circularis. The viability of cells from such inner layers is longer than those from the outer layer of the intestinal lumen (Loran and Crocker, 1963). In procedures B and C, however, the DMC obtained were likely from mucosa only because these were isolated by shaking without scraping.

In conclusion, although all three procedures evaluated in this study were capable of isolating viable DMC, the procedure using KRB-HEPES containing hyaluronidase and BSA yielded the highest number of viable DMC.

3.4 References

Ameh, D. A. and J. R. Thompson. 1990. Effect of *Escherichia coli* endotoxin on some aspects of amino acid and protein metabolism by the rat small intestine. Pages 1009-1016 in Amino Acids: Chemistry, Biology and Medicine. G. Lubec and G. A. Rosenthal, eds. Escom Science, Leiden, The Netherlands

Baldwin, R. L., and K. R. McLeod. 2000. Effects of diet forage:concentrate ratio and metabolizable energy intake on isolated rumen epithelial cell metabolism in vitro. J. Anim. Sci. 78:771–783.

Britton, R. and C. Krehbiel. 1993. Nutrient Metabolism by Gut Tissues. J. Dairy. Sci. 76:2125–2131.

Cant, J. P., B. W. McBride, and W. J. Croom. 1996. The regulation of Intestinal Metabolism and its Impact on whole animal energetics. J. Anim. Sci. 74:2541–2553.

Gross, C. J., L. M. Henderson, and D. A. Savaiano. 1986. Uptake of L-carnitine, D-carnitine and acetyl-L-carnitine by isolated guinea-pig enterocytes. Biochim. Biophys. Acta. 886:425–433.

Kaltenbach, J. P., M. H. Kaltenbach, W.B. Lyons. 1958. Nigrosin as a dye for differentiating live and dead ascites cells. Exp. Cell Res. 15:112–117.

Loran, M. R. and T. T. Crocker. 1963. Population dynamics of intestinal epithelia in the rat two months afer partial resection of the ileum. J. Cell Biol. 19:285–291.

Martinez-Hernandez, A. and P. Amenta. 1983. The basement membrane in pathology. Lab. Invest. 48:656–677.

Oba, M., R. L. Baldwin VI, and B. J. Bequette. 2004. Oxidation of glucose, glutamate, and glutamine by isolated ovine enterocytes in vitro is decreased by the presence of other metabolic fuels. J. Anim. Sci. 82:479–486.

Okine, E. K., D. R. Glimm, J. R. Thompson, and J. J. Kennelly. 1995. Influence of stage of lactation on glucose and glutamine metabolism in isolated enterocytes from dairy cattle. Metabolism. 44:325–331.

Tarkkanen, A. M., B. L. Allen, B. Westerlund, H. Holthofer, P. Kuusela, L. Risteli, S. Clegg, and T. K. Korhonen. 1990. Type V collagen as a target for type-3 fimbriae, enterobacterial adherence organelles. Mol. Microbiol. 4:1353–1361.

Underhill, C. B. 1989. The interaction of hyaluronate with the cell surface: the hyaluronate receptor and the core protein. Ciba Found Symp. 143:87–99.

Watford, M., P. Lund, and H. A. Krebs. 1978. Isolation and metabolic characteristics of rat and chicken enterocytes. Biochem. J. 178:589–596.

Wu, G. 1995. Urea synthesis in enterocytes of developing pigs. Biochem. J. 312:717–723

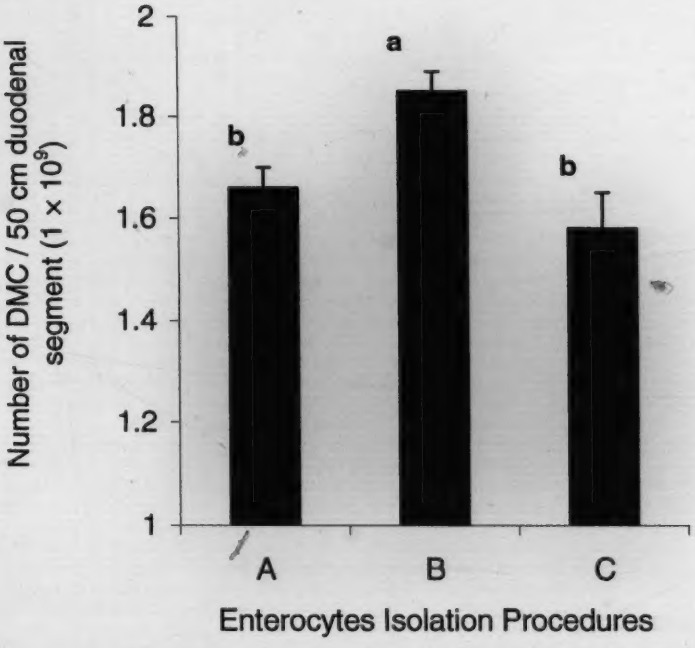


Figure 3.1. Number of viable DMC obtained immediately after slaughter per 50-cm long bovine duodenal segment in different isolation procedures (Procedure A: Duodenal mucosa was scrapped and incubated with a solution containing collagenase and dispase for 20 min, Procedure B: Duodenal segment was filled with a KRB-HEPES containing hyaluronidase and patted with finger tips, Procedure C: Duodenal segment was filled with KHB-HEPES without enzyme and incubated for 50 min. Bars with different alphabet are significantly different from one another, $P < 0.05$)

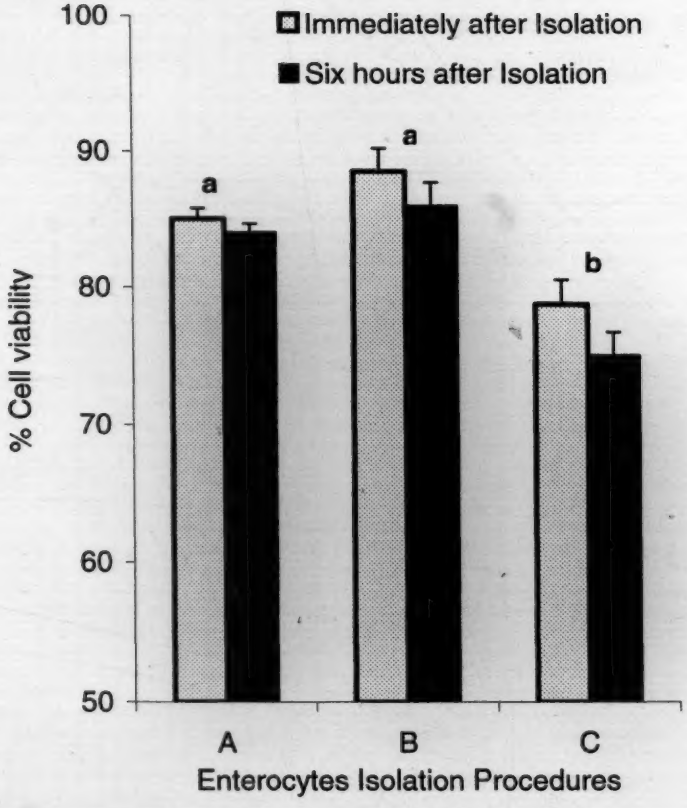


Figure 3.2. Viability of bovine DMC immediately and six hours after isolation by different isolation procedures (Procedure A: Duodenal mucosa was scrapped and incubated with a solution containing collagenase and dispase for 20 min, Procedure B: Duodenal segment was filled with a KRB-HEPES containing hyaluronidase and patted with finger tips, Procedure C: Duodenal segment was filled with KHB-HEPES without enzyme and incubated for 50 min. Cells were maintained in the incubation medium at room temperature for 6 hrs. Bars with different alphabet are significantly different from one another, $P < 0.05$)

CHAPTER 4

Effect of ammonia load on glucose metabolism by isolated ovine duodenal mucosal cells

4.1 Introduction

Glucose is one of the limiting nutrients in high producing ruminant animals (Fahey and Gerger, 1988). Gluconeogenesis, the primary source of glucose in ruminants, may not be sufficient to meet the high glucose demand especially during peak lactation in high producing dairy cows (Elliot, 1976; Hurtaud et al., 2000). Glucose absorbed in the intestine can contribute up to 30% of the total glucose supply for cows fed high concentrate diets (Huntington, 1997) and may be the significant glucose source to meet the glucose demand in lactating dairy cows. In addition, starch digestion and absorption in the small intestine is more efficient compared to starch fermentation in the rumen (Huntington et al., 2006; Owens et al., 1986). A previous study indicated that increased intestinal glucose supply decreased hepatic gluconeogenesis (Clark et al., 1977). Furthermore, enhanced glucose supply to the duodenum increased milk and milk lactose yield (Rigout et al., 2002).

Isolated ruminant duodenal mucosal cells (DMC) possess the metabolic capability to assimilate ammonia-N into non-essential amino acids (NEAA) such as alanine, glutamate and aspartate (Oba et al., 2005). Alanine synthesis can be a metabolic pathway by which DMC can detoxify ammonia because of

the net positive flux of alanine by the portal-drained viscera (El-Kadi et al., 2006) whereas aspartate and glutamate are extensively catabolized by small intestinal mucosa (Wu, 1998). Ammonia-N assimilation into alanine by DMC increased in the presence of glucose (Oba et al., 2005), but it is not known whether ammonia load affects the extent of ammonia detoxification via alanine synthesis. Although duodenal flow of ammonia varies greatly depending on the dietary CP content, its effects on glucose metabolism in DMC are not known. In addition, previous studies did not demonstrate whether glucose utilization for alanine synthesis increases when ammonia flow increased in the duodenum.

We hypothesized that glucose carbon utilized to synthesize NEAA in DMC would increase when the ruminant duodenal mucosa are exposed to greater ammonia concentrations. Thus, the objectives of the study were to evaluate the effects of ammonia load in the incubation media and those of dietary CP content on glucose metabolism in DMC isolated from growing sheep.

4.2 Materials and methods

4.2.1 Animals and diets

The experimental protocol was approved by Faculty Animal Policy and Welfare Committee at the University of Alberta (FAPWC approval # OBA-2006-32). Sheep were used as a ruminant model. Ten Suffolk crossbred

growing female sheep (36.9 ± 1.48 kg body weight, 4 months old) were fed a high CP (19.4%) or a low CP (13.1%) diet (Table 4.1) during the experiment. Total DM offered was 3.5% of the BW of each sheep and the amount was adjusted every week according to the change in BW. Animals were fed twice daily at 0800 and 1800 h. The metabolizable energy content of the diet was 3.32 and 3.39 Mcal/kg DM respectively for high and low CP diets to meet energy requirements of growing sheep (NRC, 1985). The dietary concentration of CP in high and low CP diets was determined to provide about 15% above and below the CP requirement for growing sheep, respectively (NRC, 1985).

After feeding the experimental diets for at least 3 weeks, 2 sheep were slaughtered per day for 5 days. For the first day, a lamb fed low CP diet was slaughtered at 0700 h followed by slaughter of another lamb fed high CP diet at 1400 h. To minimize the confounding effects of slaughter time, the order of slaughter was alternated with respect to treatment each day.

4.2.2 Ammonia measurement

Duodenal digesta was collected from the duodenum immediately after slaughter and supernatant was obtained after centrifuging (1000 × *g*) the digesta sample for 10 min. Ammonia concentration in the duodenal fluid was determined spectrophotometrically (Hewlett Packard 8452A Diode Array, American Laboratory Trading, Groton, CT) as described previously (Appendix 3; Fawcett and Scott, 1960).

4.2.3 Cell isolation

The DMC were isolated within 30 min after the slaughter according to a method described previously (Okine et al., 1995) with some modifications. Briefly, one-meter long duodenal segments from 50-cm distal to the pylorus were collected immediately after slaughter. The excised duodenal tissue was flushed with Krebs-Ringer HEPES buffer (NaCl 120 m*M*, KCl 4.7 m*M*, $MgSO_4$ 1.2 m*M*, KH_2PO_4 1.2 m*M*, $NaHCO_3$ 25 m*M*, and HEPES 25 m*M*; oxygenated with O_2:CO_2 [95:5], pH 7.4; solution A) to remove digesta and mucus. The flushed tissue was filled with solution A containing 2.5 mg/mL bovine serum albumin (Sigma Chemical Co., St. Louis, MO; solution B), and both ends of the tissue were clamped. The duodenal tissue filled with solution B was incubated in a container of solution A for 15 min in a shaking water bath (Julabo SW 22, Germany) at 37°C. The duodenal contents were discarded and the segment was again filled with the solution B containing 1.5 mg/mL hyaluronidase (Worthington Biochemical Corporation, Lakewood, NJ). The re-filled segments containing the digestion solution were kept on crushed ice in a container, and gently patted with fingertips for two minutes to release the DMC into the lumen. The resulting DMC suspension was washed three times with solution A followed by centrifugation (1,000 × *g* for 3 min each time). Yield of isolated cells was $98 \times 10^7 \pm 0.9 \times 10^7$ per isolation, and their viability, as determined by the trypan blue dye exclusion test (Kaltenbach, 1958), was 77.6 ± 3.2% (n = 10). Trypan blue exclusion test is the commonly used technique to determine the viability of ruminant gut epithelial cells (Baldwin and McLeod,

2000; Oba, 2004; Okine et al., 1995). In this test, cell viability is indirectly determined from cell membrane integrity. Thus, it is possible that cells' metabolic activity may have been compromised even though its membrane integrity is maintained (Strober, 1997).

4.2.4 Incubation and metabolite analysis

Immediately after cell yield and viability were determined, the isolated primary cells were incubated for 90 min with glucose (3 m*M*) and ammonium chloride (0, 0.1, 1, 5, 10, 20, or 50 m*M*) in Krebs-Ringer HEPES buffer oxygenated with O_2:CO_2 [95:5], pH 7.4 in a heated (37°C) shaking water bath (Julabo SW 22). Ammonia concentrations of the incubation media were selected to reflect the wide range of ammonia concentrations predicted to exist in the duodenum (Parker et al., 1995; Winnicka et al., 1992). The glucose concentration in the incubation media represents blood glucose concentration observed in ruminants (Fernandez et al., 2001). The study was conducted by using parallel incubations; the first set of flasks contained [2-^{13}C] glucose (Cambridge Isotope Laboratories Inc., Andover, MA) in the media to determine isotopic enrichment of non-essential amino acids and lactate, whereas, the second set of flasks contained [U-^{14}C] glucose (Sigma; 0.78 μCi/flask; 13.3 μCi/mmol of glucose) in the media to determine $^{14}CO_2$ production from glucose. Incubation was initiated by adding 0.5 mL of the cell suspension (1×10^7 viable cells) to the media in 25 mL Erlenmeyer flasks. The flasks were oxygenated and capped immediately. The flasks in the first set were capped with glass caps

whereas the flasks in the second set were sealed with a rubber serum cap fitted with a suspended center well (Kontes, Vineland, NJ) containing a folded piece of filter paper. Incubation was terminated in both sets of flasks after 90 min by adding 0.2 mL of concentrated $HClO_4$ to the media. Each treatment was carried out in triplicate, and all flasks were incubated together. Additional two sets of flasks, in triplicate, were also prepared for the assessment of endogenous metabolite concentrations and as a 0-min time control. In these cases, the media were acidified immediately after the addition of the cell suspension (endogenous metabolites) or following the addition of 3 m*M* glucose and cell suspension (0-min control).

After termination of incubation, the incubation media in the first-set flasks were neutralized with 0.275 mL K_2CO_3 (5.8 *M*), and clarified supernatant was obtained by centrifugation (2,300 × *g* for 7 min). Glucose concentration of the supernatant was determined using glucose oxidase/peroxidase enzyme (No. P7119, Sigma) and dianisidine dihydrochloride (No. F5803, Sigma) as described previously (Appendix 4; Raabo and Terkildsen, 1960), and the absorbance was determined with a microplate spectrophotometer (Spectra Max 190, Sunnyvale, CA). Net glucose disappearance was calculated by subtracting the amount of glucose in incubation media of treatment flasks from that of 0-min control.

A known weight (0.5 g) of the internal standard mixture containing tracers ([U-^{13}C] labeled alanine, aspartate, glutamate and lactate; 250 nmol/g each) was added to a known weight (2 g) of clarified medium for determination of metabolite concentrations by the isotope dilution technique. To enhance GC-MS measurements at low substrate concentrations, the internal standard mixture also contained known amounts of unlabeled alanine, aspartate, glutamate and lactate to raise unlabelled substrate concentrations to within the standard curve range. Samples were applied to 1.0 g of cation-exchange resin (AG-50W-x8, 100-200 mesh, H^+-form; Bio-Rad Lab., Hercules, CA) to separate amino acids and lactic acid. Standard curves were generated by adding different concentrations of unlabeled substrates to the internal standard. Alanine, aspartate, glutamate, and lactate were converted to tertiary butyldimethylsilyl derivatives (Calder and Smith, 1988). Concentration of metabolites and their isotopic enrichment with ^{13}C were determined using GC-MS under electron impact mode (Agilent 6890 coupled to an Agilent 5973 Mass Selective Detector, Agilent, Palo, Alto, CA). The following masses were monitored by GC-MS: alanine 260, 261, 263; lactate 261, 262, 264; aspartate 418, 419, 422; and glutamate 433, 434, 437.

For the second set of flasks, after termination of incubation, 0.3 mL of benzethonium hydroxide was injected in the center wells using syringe and left for 1 hr at room temperature to capture CO_2. Center wells were transferred to scintillation vials and filled with 4 mL of scintillant (Ecolite™, Research Product

Division, Irvine, CA). Radioactivity was determined by liquid scintillation spectroscopy (Beckman LS Beta Counter 5801, Minnesota, MN) and the amount of glucose oxidized to CO_2 was calculated after subtracting the background non-specific activity (0-min control).

4.2.5 Enzyme assay

Additional duodenal tissue (approximately 30-cm in length) was collected from the sheep immediately after slaughter. The tissue was rinsed with solution A before freezing at –80°C. Duodenal mucosal cells were scraped from the frozen duodenal tissue using a glass slide after cutting the tissue lengthwise. The scraped mucosal cells were homogenized in a buffer containing 0.15 *M* KCl, 5 m*M* $MgSO_4$ and 5 m*M* EDTA. Cell disintegration was carried out using a sonicator (Sonic 300 Dismembrator, Manassas, VA) for 3 minutes. The homogenate thus obtained was centrifuged at 21,000 × *g* at 0°C for 1 h and the supernatant was used for the measurement of hexokinase (EC 2.7.1.1) and pyruvate kinase (EC 2.7.1.40) activity as described previously by Darrow and Colowick (1962), and (Cardenas and Dyson, 1973), respectively.

4.2.6 Statistical analysis

The effect of dietary CP was determined using the following model with Mixed model procedure of SAS (version 9.1, SAS Institute, Inc., Cary, NC): $Y_{ijk} = \mu + P_i + T_j + P_i{*}T_j + \varepsilon_{ijk}$, with Y_{ijk} as the observed value, μ as the overall mean, P_i as

the effect of dietary CP concentration, and T_j as the effect of slaughter time. The linear and quadratic effects of ammonia concentration in the incubation media was determined using the following model: $Y_{ijkl} = \mu + P_i + T_j + P_i*T_j + S_k(P_i*T_j) + A_l + T_j*A_l + P_i*T_j*A_l + A_l*A_l + \varepsilon_{ijkl}$, where Y_{ijkl} was the observed value, μ was the overall mean, P_i was the effect of dietary CP concentration, T_j was the effect of slaughter time, S_k was the random effect of sheep, and A_l was the effect of ammonia concentration in the incubation media. The second statistical model had originally included interactions between effects of ammonia and dietary CP concentration, but they were removed as they were not significant for all response variables. Treatment effects were declared significant at $P < 0.05$.

4.3 Results

Dietary treatment did not affect DMI, BW gain, and ADG at slaughter (Table 4.2). The CP intake (279.1 ± 9.0 vs. 189.8 ± 3.0 g/d) and duodenal ammonia (16.4 ± 1.0 vs. 9.1 ± 1.0 m*M*) were greater for the high CP treatment compared to the low CP treatment.

Glucose disappearance was greater in the incubation media for the DMC isolated from the sheep fed the low CP diet (14.6 ± 1.6 vs. 8.6 ± 1.3 nmol / 10^6 cells / 90 min; $P < 0.05$; Table 4.3). However, dietary CP did not affect in vitro glucose carbon oxidation nor utilization for the synthesis of lactate, alanine, aspartate and glutamate.

Glucose disappearance and utilization of glucose carbon for oxidation and synthesis of aspartate or glutamate were not affected by the ammonia concentration in the incubation media. As the ammonia concentration increased, utilization of glucose carbon for lactate synthesis decreased quadratically (Table 4.4) and that for alanine synthesis decreased linearly.

Hexokinase activity was significantly higher in the duodenal mucosa collected from the sheep fed low CP diet (1.22 ± 0.06 vs. 1.04 ± 0.01 mUnit/mg protein; $P < 0.05$; Figure 4.1). However there was no effect of dietary CP on pyruvate kinase activity (Figure 4.2).

4.4 Discussion

It was observed that ammonia-N assimilation into NEAA by DMC increased in the presence of glucose (Oba et al., 2005). However, the previous study did not examine how the extent of exposure of DMC to ammonia affects glucose carbon utilization for NEAA synthesis by DMC. This information is important because glucose is one of the limiting nutrients for high producing dairy cows (Fahey and Gerger, 1988). Significant amounts of dietary starch reach the duodenum, and can be absorbed as glucose in the small intestine (Huntington, 1997; Owens et al., 1986). In addition, depending upon dietary CP concentration, the ammonia concentration in the ruminant duodenum is expected to be variable as shown in this study. Using ovine DMC as a model,

this study was conducted to determine how the extent of exposure to ammonia affects glucose metabolism by DMC.

Results of this study showed that the ammonia concentration in the incubation media did not affect glucose disappearance nor glucose carbon utilization for the production of CO_2, aspartate and glutamate by DMC, although we had hypothesized that glucose carbon utilized to synthesize NEAA in DMC would increase when the duodenal mucosa are exposed to greater ammonia concentration. Furthermore, as the ammonia concentration in the incubation media increases, glucose carbon utilization for alanine production by DMC decreased. The present results indicated that glucose provides carbon for NEAA synthesis, but glucose carbon utilization for NEAA synthesis does not increase as ammonia concentration in the incubation media increases.

The present study also showed that the DMC isolated from sheep fed a low CP diet utilized more glucose compared to those from sheep fed a high CP diet. This is attributed to greater hexokinase activity observed in the duodenal mucosa from sheep fed low dietary CP. The greater hexokinse activity indicated that sheep fed a low CP diet might have relied on glucose as a metabolic fuel to a greater extent compared with those fed a high CP diet. Although we used urea to increase dietary CP concentration, we predicted that amino acid absorption at the duodenum would be substantially greater for sheep fed the high CP diet because the amount of fermentable energy offered

in the diet should be sufficient to utilize urea-N for microbial protein synthesis (NRC, 1985). Previous studies showed that glucose metabolism in DMC can be increased when amino acids such as glutamate, glutamine (Oba et al., 2004) or leucine (Reiser et al., 1975) are limiting. Further, other studies (Castlebury and Preston, 1993; Taniguchi et al., 1995) showed that net absorption of glucose across the portal-drained viscera was reduced with decreased protein supply, indicating that low protein supply can enhance glucose demand by the gut tissues. In contrast to our finding that hexokinase is decreased with high dietary CP, hexokinase activity in rat intestinal mucosa was not affected by high dietary casein but it was increased by glucose (Shakespeare et al., 1969).

Previous in vivo studies (Castlebury and Preston, 1993; Taniguchi et al., 1995; Veira et al., 1980) showed that abomasal or duodenal protein supply increased the glucose absorption into the portal vein. In a steer infused with 800 g corn starch into the abomasum, abomasal supply of 800 g sodium caseinate increased the net glucose absorption across portal drained viscera by two fold compared to the ruminal supply of same quantity of sodium caseinate (Taniguchi et al., 1995). Similarly, in a study with sheep, small intestinal starch disappearance and glucose absorption across the portal drained viscera were increased with greater casein infusion into the abomasum (Castlebury and Preston, 1993). The reasons for the increased glucose absorption with greater CP supply in these studies could be that the

presence of additional amino acids reduced glucose metabolism in the gut tissues (Oba et al., 2004; Reiser et al., 1975). Another study also showed that abomasal infusion of casein increased Na^+ glucose co-transporter (SGLT) 1 activity in the small intestinal mucosa of sheep (Mabjeesh et al., 2003), indicating enhanced glucose absorption from the intestinal lumen.

In contrast to the results of the present study, some studies (Blouin et al., 2002; El-Kadi et al., 2006; Guerino et al., 1991) have reported that increased CP supply had either no or negative effects on net glucose absorption across portal drained viscera. Increased casein infusion into the duodenum (up to 105 g/d) had no effect on the amount of glucose absorbed from arterial circulation by mesenteric drained viscera in sheep fed a low starch (3.9%) diet (El-Kadi et al., 2006). In this study, glucose absorption in the duodenum was expected to be negligible because of the extremely low dietary starch intake, which might be the reason for lack of protein effects on glucose metabolism. Intestinal protein supply affected glucose absorption across portal drained viscera when there was large quantity of starch available in the duodenum (Richards et al., 2002; Taniguchi et al., 1995; Veira et al., 1980). In another study with dairy cows, increased MP (1930 vs. 1654 g/d) had no effect on net absorption of glucose in portal drained viscera (Blouin et al., 2002). Similarly, in a study with steers fed high concentrate (78% corn) diet, abomasal infusion of casein (150 g and 300 g /d) decreased the amount of glucose absorbed across the portal

drained viscera (Guerino et al., 1991). The exact mechanism responsible for the discrepancies among the literature warrants further investigation.

In the present study, dietary CP intake had no effect on amount of glucose oxidized, and glucose carbon utilized for lactate, alanine, aspartate and glutamate production in the isolated DMC. Thus, the metabolic pathways that account for greater in vitro glucose disappearance by DMC for the low CP treatment were not identified in the current study. However, the greater glucose disappearance might be attributed its utilization to supply the pentose phosphate pathway, one of the major metabolic pathways in enterocytes (Wu, 1996).

In conclusion, enhanced ammonia load in the incubation media does not increase glucose utilization by DMC, and glucose carbon does not play a significant role in assimilation of ammonia-N into alanine, aspartate, or glutamate when the ammonia concentration in the media increases. High dietary CP intake reduced glucose metabolism in DMC probably because of decreased hexokinase activity. Further investigation is warranted to understand the physiological basis that regulates glucose utilization in the DMC.

4.5 References

Blouin, J. P., J. F. Bernier, C. K. Reynolds, G. E. Lobley, P. Dubreuil, and H. Lapierre. 2002. Effect of supply of metabolizable protein on splanchnic fluxes of nutrients and hormones in lactating dairy cows. J. Dairy Sci. 85:2618–2630.

Calder, A. G. and A. Smith. 1988. Stable isotope ratio analysis of leucine and ketoisocaproic acid in blood plasma by gas chromatography/mass spectrometry: Use of tertiary butyldimethylsilyl derivatives. Rapid Commun. Mass Spectrom. 2:4–16.

Cardenas, J. M. and R. D. Dyson. 1973. Bovine pyruvate kinase. II. Purification of the liver isozyme and its hybridization with skeletal muscle pyruvate kinase. J. Biol. Chem. 248:6938–6944.

Castlebury, R. E. and R. L. Preston. 1993. Effect of dietary protein level on nutrient digestion in lambs duodenally infused with cornstarch. J. Anim. Sci. 71(Suppl. 1):264. (Abstr.)

Clark, J. H., H. R. Spires, R. G. Derrig, and M. R. Bennink. 1977. Milk production, nitrogen utilization and glucose synthesis in lactating cows infused postruminally with sodium caseinate and glucose. J. Nutr. 107:631–644.

Darrow, R. A. and S. P. Colowick. 1962. Hexokinase from baker's yeast. Pages 226–227 in Methods in Enzymology. Vol. 5. S. P. Colowick and N. O. Kaplan, eds. Academic Press Inc., NY.

Kaltenbach, J. P., M. H. Kaltenbach, W.B. Lyons. 1958. Nigrosin as a dye for differentiating live and dead ascites cells. Exp. Cell Res. 15:112–117.

El-Kadi, S. W., R. L. Bladwin VI, N. E. Sunny, S. L. Owens, and B. J. Bequette. 2006. Intestinal protein supply alters amino acid, but not glucose, metabolism by the sheep gastrointestinal tract. J. Nutr. 136:1261–1269.

Elliot, J. M. 1976. The glucose economy of the lactating dairy cow. Proc. Cornell Nutr. Conf. Feed Manuf., Ithaca, NY:59–66.

Fawcett, J. K. and J. E. Scott. 1960. A rapid and precise method for the determination of urea. J. Clin. Path. 13:156–160.

Fahey Jr., G. C. and L. L. Gerger. 1988. Carbohydrate nutrition of ruminants. Prentice Hall, Englewood Cliffs, NJ.

Fernandez, J. M., T. Sahlu, S. P. Hart, M. J. Potchoiba, H. M. E. Shaer, N. Jacquemet, and H. Carneiro. 2001. Experimentally-induced subclinical hyperammonemia in dairy goats. Small Ruminant Research. 42:5–20.

Guerino, F., G. B. Huntington, and R. A. Erdman. 1991. The net portal and hepatic flux of metabolites and oxygen consumption in growing beef steers given postruminal casein. J. Anim. Sci. 69:387–395.

Huntington, G. B. 1997. Starch utilization by ruminants: From basics to the bunk. J. Anim. Sci. 75:852–867.

Huntington, G. B., D. L. Harmon, and C. J. Richards. 2006. Sites, rates, and limits of starch digestion and glucose metabolism in growing cattle. J. Anim. Sci. 84 (E. Suppl.):E14–E24.

Hurtaud, C., S. Lemosquet, and H. Rulquin. 2000. Effect of graded duodenal infusions of glucose on yield and composition of milk from dairy cows. 2. Diets based on grass silage. J. Dairy Sci. 83:2952–2962.

Mabjeesh, S. J., D. Guy, and D. Sklan. 2003. Na+/glucose co-transporter abundance and activity in the small intestine of lambs: enhancement by abomasal infusion of casein. Br. J. Nutr. 89:573–580.

NRC. 1985. Nutrient Requirements of Sheep. 6th revised ed. National Research Council, Washington, D.C.

Oba, M., R. L. Baldwin VI, and B. J. Bequette. 2004. Oxidation of glucose, glutamate, and glutamine by isolated ovine enterocytes in vitro is decreased by the presence of other metabolic fuels. J. Anim. Sci. 82:479–486.

Oba, M., R. L. Baldwin VI, S. L. Owens, and B. J. Bequette. 2005. Metabolic fates of ammonia in ruminal epithelial and duodenal mucosal cells isolated from growing sheep. J. Dairy Sci. 88:3963–3970.

Okine, E. K., D. R. Glimm, J. R. Thompson, and J. J. Kennelly. 1995. Influence of stage of lactation on glucose and glutamine metabolism in isolated enterocytes from dairy cattle. Metabolism. 44:325–331.

Owens, F. N., R. A. Zinn, and Y. K. Kim. 1986. Limits to starch digestion in the ruminant small intestine. J. Anim. Sci. 63:1634–1648.

Parker, D. S., M. A. Lomax, C. J. Seal, and J. C. Wilton. 1995. Metabolic implications of ammonia production in the ruminants. Proc. Nutr. Soc. 54:549–563.

Raabo, E., and T. C. Terkildsen. 1960. On the enzymatic determination of blood glucose. Scand. J. Clin. Lab. Invest. 12:402–407.

Reiser, S., O. E. Michaelis 4th, and J. Hallfrisch. 1975. Effects of sugars on leucine and lysine uptake by intestinal cells from rats fed sucrose and stock diets. Proc. Soc. Exp. Biol. Med., 1975. Available: http://www.ebmonline.org/ cgi/content /abstract/150/1/110. Accessed Jan. 10, 2007.

Richards, C. J., A. F. Branco, D. W. Bohnert, G. B. Huntington, M. Macari, and D. L. Harmon. 2002. Intestinal starch disappearance increased in steers abomasally infused with starch and protein. J. Anim. Sci. 2080:3361–3368.

Rigout, S., S. Lemosquet, J. E. Van Eys, J. W. Blum, and H. Rulquin. 2002. Duodenal glucose increases glucose fluxes and lactose synthesis in grass silage-fed dairy cows. J. Dairy Sci. 85:595–606.

Shakespeare, P., L. M. Srivastava, and G. Hubscher. 1969. Glucose metabolism in the mucosa of the small intestine: the effect of glucose on hexokinase activity. Biochem. J. 111:63–67.

Strober, W. 1997. Trypan blue exclusion test of cell viability. In Current Protocols in Immunololgy. Coligan, J.E., A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober (eds.) John Wiley & Sons, Inc. Hoboken, NJ

Taniguchi, K., G. B. Huntington, and B. P. Glenn. 1995. Net nutrient flux by visceral tissues of beef steers given abomasal and ruminal infusions of casein and starch. J. Anim. Sci. 73:236–249.

Veira, D. M., G. K. MacLoed, J. H. Burton, and J. B. Stone. 1980. Nutrition of the weaned Holstein calf. II. Effect of dietary protein level on nitrogen balance, digestibility and feed intake. J. Anim. Sci. 50:945–951.

Winnicka, A. W., K. P. Ostaszewski, E. Miernik-Degnrska, and A. Degnrski. 1992. Effect of subclinical hyperammonaemia on phagocytic activity of polymorphonuclear leukocytes isolated from peripheral blood of sheep. Small Ruminant Research. 7:253–264.

Wu, G. 1996. An important role of pentose cycle in the synthesis of citrulline and proline from glutamine in porcine enterocytes. Arch. Biochem. Biophys. 336:224–230.

Wu, G. 1998. Intestinal mucosal amino acid catabolism. J. Nutr. 128:1249–1252.

Table 4.1 Ingredients and nutrient composition of the experimental diets

Item	Diet	
	High CP	Low CP
Diet Ingredients	(% of DM)	
Dry ground corn grain	24.4	24.5
Beet pulp	51.3	53.5
Alfalfa pellet	21.3	21.3
Urea	2.28	0
Fort salt[1]	0.38	0.38
Dicalcium phosphate[2]	0.36	0.36
Vitamin ADE[3]	0.03	0.03
Nutrient Composition	(% of DM)	
DM, %	90.1	90.4
CP	19.4	13.1
ADF	22.5	21.4
NDF	33.5	32.4
Ash	9.2	8.1
Ca	1.1	1.1
P	0.2	0.2
Metabolizable energy, Mcal/kg	3.32	3.39

[1]contained 10% Mg, 6% Zn, 4.5% Fe, 1% Mn, 0.5% Cu, 0.3% I and 0.05% Co.

[2]contained 19.5% Ca, 18.5% P, 0.18% (max) F, and 1.4% (max) Fe.

[3]contained 1500 IU vitamin A, 3000 IU vitamin D, 3.7 IU vitamin E per g.

Table 4.2 Effects of dietary CP level on the performance of growing sheep

Item	Diet		SE	*P* value
	High CP	Low CP		
DMI, kg/d	1.46	1.45	0.03	0.87
CP intake, g/d	279.1	189.6	6.68	<0.001
ADG, g/d	363.0	247.2	74.26	0.33
BW before slaughter, kg	46.0	44.0	0.95	0.17
Duodenal ammonia, m*M*	16.4	9.1	0.9	0.005

Table 4.3 Effects of dietary CP level on glucose utilization by the isolated duodenal mucosal cells in vitro

Item	Diet		SE	*P* value
	High CP	Low CP		
	— (nmoles glucose / 10^6 cells / 90 min)—			
Disappearance	8.6	14.6	1.49	0.03
Oxidation	0.30	0.23	0.05	0.40
Utilization for synthesis:				
Lactate	3.94	5.68	0.93	0.13
Alanine	0.19	0.23	0.03	0.38
Aspartate	0.09	0.09	0.04	0.89
Glutamate	0.70	0.56	0.14	0.52

Table 4.4 Effects of ammonia concentration in the incubation media on glucose utilization by the isolated duodenal mucosal cells in vitro

Item	Ammonia concentration (m*M*)							SE	P value	
	0	0.1	1	5	10	20	50		L[1]	Q[2]
	(nmoles glucose / 10^6 cells / 90 min)									
Disappearance	8.24	11.10	11.22	8.48	11.86	11.32	18.75	2.75	0.65	0.77
Oxidation	0.28	0.30	0.23	0.29	0.28	0.28	0.20	0.05	0.96	0.61
Utilization for synthesis:										
Lactate	5.85	6.50	6.30	5.75	5.48	4.94	4.92	0.30	< 0.001	0.05
Alanine	0.24	0.23	0.24	0.22	0.19	0.16	0.20	0.04	0.03	0.07
Aspartate	0.09	0.12	0.11	0.09	0.08	0.09	0.06	0.03	0.47	0.77
Glutamate	0.49	0.71	0.95	0.76	0.58	0.31	0.63	0.19	0.08	0.11

[1]Linear effect of ammonia concentration in the incubation media

[2]Quadratic effect of ammonia concentration in the incubation media

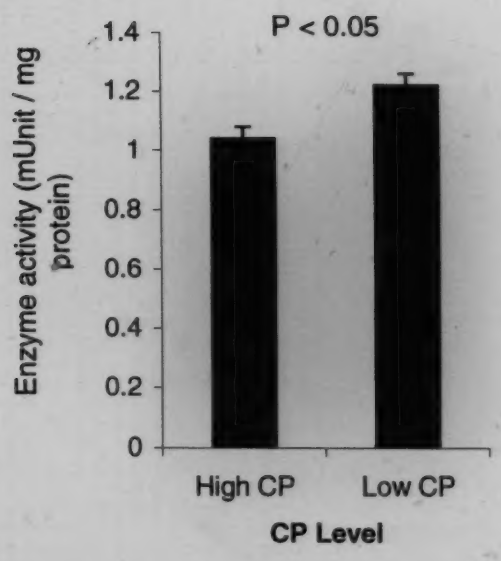


Figure 4.1. Hexokinase activity in the duodenal mucosa of growing female sheep fed low (13.1%) and high (19.4 %) CP diets

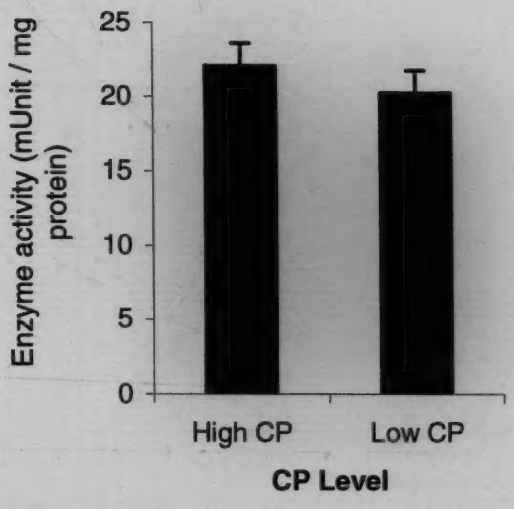


Figure 4.2. Pyruvate kinase activity in the duodenal mucosa of growing female sheep fed low (13.1%) and high (19.4 %) CP diets

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

5.1 General discussion

Nutrient metabolism in the duodenal mucosa plays a vital role in the absorption of digested nutrients from the gut lumen. Previous studies reported that the duodenal mucosa possessed the metabolic capability to utilize ammonia-N for the synthesis of NEAA (Oba et al., 2005; Wu, 1998). The presence of glucose enhanced the ammonia-N assimilation into amino-N in ruminant isolated DMC (Oba et al., 2005). This study aimed at understanding glucose metabolism in the DMC at varying concentrations of ammonia. A preliminary experiment was also conducted to determine the efficacy of the procedures described in the literature for the isolation of ruminant DMC to be used for in vitro experiments.

In the DMC isolation study, three cell isolation procedures differing in enzymatic and incubation methods were compared. The results of this study demonstrated that the use of hyaluronidase along with shaking can isolate the highest number of total viable cells per unit of duodenal tissue compared to the other two procedures evaluated in this study. The results will be useful for future research experiments in ruminant nutrition and metabolism involving short-term incubation of the DMC.

Use of primary mucosal cell is not always practical in metabolic experiments. The primary cell suspension might not be suitable for the experiments involving longer incubation time because of the loss of viability. In addition, animals have to be sacrificed each time prior to the experiment to obtain freshly isolated cells. Therefore, future research focus should be directed towards developing a ruminant DMC cell line for in vitro studies.

In the glucose metabolism study, it was hypothesized that DMC would increase glucose carbon utilization for assimilation of ammonia-N into NEAA when the ammonia concentration in the incubation media increased. The results indicate that glucose provides carbon for NEAA synthesis, but glucose does not serve as a carbon source to maximize ammonia-N assimilation to amino-N at greater ammonia concentrations. Other nutrients such as glutamate, glutamine, propionate and arginine present in the duodenum might be involved in the assimilation of ammonia-N to amino-N at high ammonia concentrations. Therefore research on the role of these substrates on ammonia-N utilization by the ruminant duodenal mucosa will be useful for improving ammonia utilization efficiency in ruminants.

The present results also demonstrated that glucose disappearance was lower in the DMC isolated from sheep fed a high CP diet compared to that from sheep fed a low CP diet. Some in vivo studies reported that greater intestinal protein supply increased glucose absorption across the PDV (Castlebury and

Preston, 1993; Taniguchi et al., 1995) but other studies did not observe such an increase in glucose absorption resulting from greater intestinal protein supply (Guerino et al., 1991; Blouin et al., 2002). It is possible that different pathways for glucose metabolism become dominant at different physiological and dietary conditions. Further, in the present in vitro study, both the apical and basolateral membranes of the isolated DMC are exposed to the same concentration of the substrates present in the incubation media. This may not be the case under normal physiological environment of the duodenal mucosa in vivo. Therefore, to understand the discrepancy of the results among these studies, further studies on the regulatory mechanism of glucose metabolism in the ruminant GI tract are necessary.

5.2 Future research

Identifying the specific physiological reasons for greater glucose utilization in the DMC obtained from the low dietary CP treatment was beyond the scope of this study. Further investigation on the mechanism of glucose utilization in the duodenum of high yielding dairy cows will be useful to improve glucose supply in early lactation. Three important aspects of intestinal glucose utilization have to be studied in detail. Firstly, in order to increase the digestion of the escape starch in the small intestine, it is important to determine the effect of ammonia and VFA on the production and activity of pancreatic α-amylase and brush border carbohydrases in high yielding dairy cows. Secondly, to increase

glucose absorption across the PDV, it would be useful to evaluate the effects on glucose transporter activity in lactating cows' DMC varying in concentrates of substrates such as starch, ammonia and VFA in the duodenal lumen. Finally, to identify the possible glucose metabolic pathways in the ruminant duodenal mucosa, the activity of enzymes needed for glucose metabolism in ruminant DMC at varying concentrations of substrates should be studied.

Pentose phosphate pathway is a significant glucose metabolism pathway in non ruminant enterocytes (Wu, 1996), however no study has been done to evaluate the glucose metabolism via this pathway in ruminants gut tissues. Further research is needed to determine the role of Pentose phosphate pathway for glucose utilization in ruminant DMC, could be helpful to increase intestinal glucose absorption in ruminants.

The data from the present study indicate that glucose does not serve as a carbon source for increased NEAA synthesis during high ammonia load in ruminant DMC. Therefore, studies on the role of the substrates such as propionate and glutamate on ammonia-N utilization by the duodenal mucosa will be useful to identify the carbon source required for the NEAA synthesis at the higher ammonia load. Once the substrate that increases ammonia assimilation to amino-N at high ammonia concentrations is identified by an in vitro study, an in vivo study should be designed to see whether the results could be replicated in the animal system. The results from these experiments

could be used to formulate strategies that might improve N utilization efficiency in ruminants.

5.3 Conclusion

The results from the DMC isolation study indicate that viable ruminant DMC required for short term in vitro studies can be isolated with or without using tissue disassociation enzymes. Hyaluronidase treatment resulted in the highest number of viable DMC. The glucose metabolism study indicated that enhanced ammonia load in the incubation media does not increase glucose utilization by DMC, and that glucose carbon does not play significant role in assimilation of ammonia-N into alanine, aspartate, or glutamate when the ammonia concentration in the media increases. The results also demonstrate that high dietary CP reduces glucose metabolism in DMC probably because of decreased hexokinase activity.

5.5 References

Blouin, J. P., J. F. Bernier, C. K. Reynolds, G. E. Lobley, P. Dubreuil, and H. Lapierre. 2002. Effect of supply of metabolizable protein on splanchnic fluxes of nutrients and hormones in lactating dairy cows. J. Dairy Sci. 85:2618–2630.

Castlebury, R. E., and R. L. Preston. 1993. Effect of dietary protein level on nutrient digestion in lambs duodenally infused with cornstarch. J. Anim. Sci. 71(Suppl. 1): 264. (Abstr.)

Guerino, F., G. B. Huntington, and R. A. Erdman. 1991. The net portal and hepatic flux of metabolites and oxygen consumption in growing beef steers given postruminal casein. J. Anim. Sci. 69:387–395.

Mabjeesh, S. J., D. Guy, and D. Sklan. 2003. Na^+ glucose co-transporter abundance and activity in the small intestine of lambs: Enhancement by abomasal infusion of casein. Br. J. Nutr. 89:573–580.

NRC. 1985. Nutrient requirements of sheep. 6th revised ed. National Research Council, Washington, D.C.

Oba, M., R. L. Baldwin VI, S. L. Owens, and B. J. Bequette. 2005. Metabolic fates of ammonia in ruminal epithelial and duodenal mucosal cells isolated from growing sheep. J. Dairy Sci. 88: 3963–3970.

Taniguchi, K., G. B. Huntington, and B. P. Glenn. 1995. Net nutrient flux by visceral tissues of beef steers given abomasal and ruminal infusions of casein and starch. J. Anim. Sci. 73: 236–249.

Wu, G. 1996. An important role of pentose cycle in the synthesis of citrulline and proline from glutamine in porcine enterocytes. Arch. Biochem. Biophys. 336: 224–230.

Wu, G. 1998. Intestinal mucosal amino acid catabolism. J. Nutr. 128: 1249–1252.

APPENDIX

A 1. Enzymatic assay of pyruvate kinase

Conditions: Temp = 37°C, pH = 7.6, Absorbance = 340 nm, Light path = 1 cm

Method: Continuous Spectrophotometric Rate Determination

Reagents:

A. **100 m*M* Potassium Phosphate Buffer, pH 7.6 at 37°C.**
Prepare 100 mL in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Adjust to pH 7.6 at 37°C with 1 M KOH.

B. **8.0 m*M* Phospho(enol)pyruvate Solution (PEP)**
Prepare 1 mL in deionized water using Phospho(enol)Pyruvate, Monopotassium Salt.

C. **3 m*M* ß-Nicotinamide Adenine Dinucleotide, Reduced Form Solution (ß-NADH)**
Dissolve the contents of a 10 mg vial of ß- Nicotinamide Adenine Dinucleotide, Reduced Form, Disodium Salt, in the appropriate volume of Reagent A. Prepare fresh.

D. **100 m*M* Magnesium Sulfate Solution**
Prepare 1 mL in deionized water using Magnesium Sulfate, Heptahydrate.

E. **40 m*M* Adenosine Diphosphate Solution (ADP)**
Prepare 1 mL in deionized water using Adenosine 5'-Diphosphate, Di(Monocyclohexylammonium) Salt.

F. **L-Lactic Dehydrogenase Solution (LDH)**
Immediately before use, prepare a solution containing 500 units/mL of L-Lactic Dehydrogenase, in cold Reagent A.

G. **30 m*M* Fructose 1,6-Diphosphate Solution (F 1,6-P)**
Prepare 2 mL in deionized water using Fructose 1,6- Diphosphate, Sodium Salt.

H. **Pyruvate Kinase**
Immediately before use, prepare a solution containing 0.3 - 0.6 unit/mL of Pyruvate Kinase in cold Reagent A.

Procedure:

Pipette (in mL) the following reagents into suitable cuvettes:

	Test	Blank
Deionized Water	1.30	1.30
Reagent A (Buffer)	0.80	0.90
Reagent B (PEP)	0.16	0.16
Reagent C (ß-NADH)	0.20	0.20
Reagent D (Magnesium Sulfate)	0.20	0.20
Reagent E (ADP)	0.10	0.10
Reagent F (LDH)	0.04	0.04
Reagent G (F 1,6-P)	0.10	0.10

Mix by inversion and equilibrate to 37°C. Monitor the A340 nm until constant, using a suitably thermostatically controlled spectrophotometer. Then add:

Reagent H (Enzyme Solution)	0.10	------

Immediately mix by inversion and record the decrease in A340 nm for approximately 5 minutes. Obtain the Δ A340 nm/min using the maximum linear rate for both the Test and Blank.

Calculations:

$$\text{Units/mL enzyme} = \frac{(\Delta \text{ A340 nm/min Test} - \Delta \text{ A340 nm/min Blank})(3)(df)}{(6.22)(0.1)}$$

3 = Total volume (in mL) of assay
df = Dilution factor
6.22 = Millimolar extinction coefficient of ß-NADH at 340 nm
0.1 = Volume (in mL) of enzyme used

$$\text{Units/mg solid} = \frac{\text{units mL/ enzyme}}{\text{mg solid/mL enzyme}}$$

$$\text{Units/mg protein} = \frac{\text{units/mL enzyme}}{\text{mg protein/mL enzyme}}$$

Unit definition:

One unit will convert 1.0 µmole of phospho(enol)pyruvate to pyruvate per minute at pH 7.6 at 37°C in the presence of 1.0 m*M* of fructose 1,6-diphosphate. In the absence of added fructose 1,6-diphosphate which acts as an (activator), considerably lower activity will be observed.

Reference:

Cardenas, J. M. and R. D. Dyson. 1973. Bovine pyruvate kinase. II. Purification of the liver isozyme and its hybridization with skeletal muscle pyruvate kinase. J. Biol. Chem. 248:6938–6944.

A 2. Enzymatic assay of hexokinase

Conditions: T = 25°C, pH 8.5, A560nm, Light path = 1 cm

Method: Continuous Spectrophotometric Rate Determination

Reagents:

A. **100 m*M* Glycylglycine Buffer, pH 8.5 at 25°C**
prepare 100 mL in deionized water using Glycylglycine, Free base, Adjust to pH 8.5 at 25°C with 1 M HCl

B. **200 m*M* Adenosine 5'-Triphosphate Solution (ATP)**
Prepare fresh 10 mL solution in deionized water using Adenosine 5'-Triphosphate, Disodium Salt.

C. **200 m*M* Glucose Solution (Gluc)**
Prepare 10 mL in deionized water using ß-(+)-Glucose.

D. **0.01% Cresol Red with 128 m*M* Magnesium Chloride Solution (Cresol Red)** Prepare 200 mL in deionized water using Cresol Red, Sodium Salt, and Magnesium Chloride, Hexahydrate. Facilitate by first dissolving Cresol Red into 6.6 mL of 95% ethanol. Transfer this solution to a 200 mL graduated cylinder and add 5.2 g of Magnesium Chloride, Hexahydrate. Dilute to 200 mL with deionized water.

E. **100 m*M* Hydrochloric Acid Standardized Solution (HCl)**
Prepare 1 liter in deionized water using Concentrated Hydrochloric Acid. Standardize against Tris Base with Sigma 121 indicator. Color change is from orange to pink.

F. **0.5% Glucose Solution**
Prepare 50 mL using ß-D-Glucose.

Sample solution: prepare a sample solution containing hexokinase enzyme.

Procedure:
Prepare a reaction cocktail by pipetting (in mL) the following reagents into a suitable container:

Reagent B (ATP)	5.00
Reagent D (Cresol Red)	6.60

Mix and slowly add 0.1 M NaOH until the solution just turns from red to purple (pH about 8.2). Then add:

Deionized Water	33.40
Reagent A (Buffer)	5.00

Mix. Adjust to pH 8.5 at 25°C with 100 m*M* HCl or 100 m*M* NaOH, if necessary.

Titer Determination:

Determine titer of reaction cocktail by pipetting (in mL) the following reagents into a suitable cuvette:

Reaction Cocktail	2.50
Reagent C (Glucose)	0.40

Mix by inversion and equilibrate to 25°C. Monitor the A560 nm until constant, using a suitably thermostatically controlled spectrophotometer. Record the initial A560 nm. Then add:

Reagent D (HCl)	0.10

Mix and immediately record final A560 nm.

$$\text{Titer} = \frac{(\text{A560 nm initial} - \text{A560 nm final})\ (2.9)}{(1000)\ (0.1)\ (\text{Molarity of Reagent D})}$$

2.9 = Volume of Titer reaction Mix
1000 = Coversion from millimolar to micromolar
0.1 = Volume of Reagent D used

Sample: Pipette (in mL) the following reagents into suitable cuvettes:

	Test	Blank
Reaction Cocktail	2.50	2.50
Reagent C (Gluc)	0.40	0.40

Mix by inversion and equilibrate to 25°C. Monitor the A_{560}nm until constant, using a suitably thermostatically controlled spectrophotometer. Then add:

Reagent F (Diluent)	------	0.10
Reagent G (Enzyme Solution)	0.10	------

Immediately mix by inversion and record the decrease in A560 nm for approximately 5 minutes. Obtain the ΔA560 nm/minute using the maximum linear rate for both the Test and Blank.

Calculations:

$$\text{Units/mg enzyme} = \frac{\text{A560 nm/min Test} - \text{A560 nm/min Blank}}{(\text{titer})\ (\text{mg enzyme/mL RM})}$$

RM = Reaction Mix

Unit Definition:
One unit will phosphorylate 1.0 µmole of glucose per minute at pH 8.5 (± 0.5) at 25°C.

Reference

Darrow, R. A. and S. P. Colowick. 1962. Hexokinase from baker's yeast. Pages 226–227 in Methods in Enzymology. Vol. 5. S. P. Colowick and N. O. Kaplan, eds. Academic Press Inc., NY.

Partridge, N.C., Hoh, C.H., Weaver, P.K. and Oliver, I.T. 1975. Premature induction of glucokinase in the nonatal rat by thyroid hormone. Eur. J. Biochem. 51: 49–54

A 3. Ammonia-N determination

1. Reagents:

a) Sodium phenate

12.5 g phenol + 6.2 g NaOH in 500 mL volumetric flask (dissolved and made to volume with deoinized water)

b) Sodium Nitroprusside

Stock solution (1%) – 1 g/100 mL H_2O

Working solution (0.01%) – 5 mL stock diluted to 500 mL (vol. flask) with deionized water

Note: working solution should be prepared fresh daily

c) Sodium Hypochlorite

0.02 N 15 mL NaOCl (4-6%) diluted to 500 mL with deionized water

Note: the pH should be adjusted to 12.0 with 50%NaOH

NOTE: All solutions should be stored in the fridge with tin foil wrapped around the containers. The solutions should be brought to room temperature before use.

2. Standard solution: 100 ug NH_3-N/mL

0.4716 g Ammonium sulfate (dried at 60°C for 2 hrs) /L deionized water

The pH should be close to that of samples

3. Sample preparation

Duodenal Digesta

- thaw and mix well
- transfer around 5 mL to 16 x 100 mm glass tubes and centrifuge for 10 min in the bench top centrifuge at maximum setting (100 x g)
- pipette 40 µl aliquots into other 16 x 100 glass test tubes (each sample is done in duplicate)

4. Colour Development

a) Prepare a standard curve by pipetting 0, 10, 30, 40, 50, 60 µl from standard solution (in duplicate) into 16 x 100mm glass tubes. Standards must be run with every set (approximately 40 tubes) (for the "0", pipette 20 µl distilled water). A control sample should be run in duplicate with each standard curve.

b) Add reagents to the standards and samples in the following order

- 2 mL phenate solutions and vortex
- 3 mL nitroprusside solution and vortex

- 3 mL hypochlorite, cover tube with parafilim and invert several times (deliver with brown bottle dispensers set to the appropriate volumes).

c) Develop colour at room temperature in the dark for 1hour (put tubes into a cupboard).
d) Read absorbance on dipping probe at 600 nm using distilled water to zero spectrophotometer. (Be sure to mix sample by inversion before reading absorbance).

NOTE: Keep the sample covered with parafilm at all times. Ammonia tends to be absorbed from the air. When dispensing the solutions, be consistent as possible with the volume because the reading can vary (i.e. first drips cause a problem)

Calculations:

1. Run regression using 0, 1, 2, 3, 4, 5, and 6 as x and absorbances as y.
2. µg/volume added to tube = $\frac{\text{Absorbance - Intercept}}{\text{Slope}}$

Dry sample = DM at 105°C x DM at 60°C x 0.001
Conc. mgN = average ug/tube x (1000/volume added) x 0.001 (i.e., ug to mg, (mgN/ mL)
Total N = conc. mgN x (1000-dry sample)
gm NH_3/kg sample = total N/ dry sample

NOTE: [(maximum- minimum)/average) x 100] < 10 percent is acceptable.

Reference:

Fawcett, J. K., and J. E. Scott. 1960. A rapid and precise method for the determination of urea. J. Clin. Path. 13:156–160.

A 4. Glucose determination

1) Solution A
Dissolve the contents of 2 capsules of PGO Enzyme preparation into 100 mL of dH2O into an amber flask

2) Solution B
Using the squirt bottle, add 20 g ± 0.01 g of dH_2O into the vial of dianisidine dihydrochloride.

3) Solution AB
Add 3.2 mL of solution B to 100 mL of solution A. (Can keep solution upto 3 weeks in refrigerator)

Procedure

1) Prepare a standard glucose curve into the test tube using the stock solution in the glucose kit:

mg Glucose /100 mL	0	20	40	60	80	100
Stock solution (µl)	0	200	400	600	800	1000
dH2O (µl)	1000	800	600	400	200	0

2) Use a plate with flat- bottom wells

3) Using plate wells A1 and H1, A2 and H2, etc., through A6 and H6, pipette a 10 µl aliquot of each standard curve solution. Two samples, therefore of each standard will be read by plate reader. Make duplicate plate for each series of samples. Be sure to include duplicate samples of starch standard and in house standard to each plate.

4) Pipette a 10 µl aliquot of each sample into remaining wells.

5) Add 300 µl solution AB to each well with a multi-channel micropipette, turn the plate reader on and shake the plate with the plate reader for at least 10 sec. Allow to sit at room temperature for 45 min covered with the tin foil.

6) Read the absorbance of samples at 450 nm.

Calculate regression equation. Using the regression equation, calculate mg glucose per 100 mL for all samples.

Reference:

Raabo, E., and T. C. Terkildsen. 1960. On the enzymatic determination of blood glucose. Scand. J. Clin. Lab. Invest. 12:402–407.